Comparison of SARS-CoV-2 evolution in vitro during passage in Vero and primary human airway cells

Collaboration Between Host and Viral Factors Shape SARS-CoV-2 Evolution

Connor G. G. Bamford^{1*}, Lindsay Broadbent¹, Elihu Aranday-Cortes², Mary McCabe¹, James McKenna³, David Courtney¹, Olivier Touzelet¹, Ahlam Ali^{1,4}, Grace Roberts¹, Guillermo Lopez Campos¹, David Simpson¹, Conall McCaughey³, Derek Fairley³, Ken Mills⁴, Ultan F. Power^{1*}, and the Breathing Together Investigators[±]

¹ Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, UK.

² Medical Research Council-University of Glasgow Centre for Virus Research, University of Glasgow, Glasgow, UK.

³ Regional Virus Laboratory, Belfast Health and Social Care Trust, Belfast, UK

⁴ Patrick. G. Johnston Centre for Cancer Research, Queen's University Belfast, Belfast, UK.

*co-corresponding authors

Connor G. G. Bamford email: c.bamford@qub.ac.uk

Ultan F. Power email: u.power@qub.ac.uk

± = Breathing Together investigators: Professor Andrew Bush, Professor Adnan Custovic, Professor Peter Ghazal, Dr. Mindy Gore, Professor Jonathan Grigg, Professor Clare M. Lloyd, Professor Benjamin Marsland, Professor Ultan Power, Professor Graham Roberts, Professor Sejal Saglani, Professor Jurgen Schwarze, Professor Mike Shields, and Professor Steve Turner.

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Abstract

SARS-CoV-2 continues to evolve, resulting in several ‘variants of concern’ with novel properties. The factors driving SARS-CoV-2 fitness and evolution in the human respiratory tract remain poorly defined. Here, we provide evidence that both viral and host factors co-operate to shape SARS-CoV-2 genotypic and phenotypic change. Through viral whole-genome sequencing, we explored the evolution of two clinical isolates of SARS-CoV-2 during passage in unmodified Vero-derived cell lines and in parallel, in well-differentiated primary nasal epithelial cell (WD-PNEC) cultures. We identify a consistent, rich genetic diversity arising in vitro, variants of which could rapidly rise to near-fixation with 2 passages. Within isolates, SARS-CoV-2 evolution was dependent on host cells, with Vero-derived cells facilitating more profound genetic changes. However, most mutations were not shared between strains. Furthermore, comparison of both Vero-grown isolates on WD-PNECs disclosed marked growth attenuation mapping to the loss of the polybasic cleavage site (PBCS) in Spike while the strain with mutations in NSP12 (T293I), Spike (P812R) and a truncation of ORF7a remained viable in WD-PNECs. Our work highlights the significant genetic plasticity of SARS-CoV-2 while uncovering an influential role for collaboration between viral and host cell factors in shaping viral evolution and fitness in human respiratory epithelium.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Family: *Coronaviridae*; Genus: *Betacoronavirus*) has a positive-sense, non-segmented, single-stranded RNA genome of ~30,000 nucleotides in length (**Lu et al., 2020; Wu et al., 2020**). The SARS-CoV-2 genome encodes at least 29 proteins, expressed from translation of a 5' major open reading frame (ORF1ab), including Nsp3 and Nsp12 (viral RNA-dependent RNA polymerase), and a series of nested transcripts at the 3' terminus, including Spike (S; the viral attachment and fusion glycoprotein) and ORF7a. SARS-CoV-2 emerged into the human population in late 2019, causing coronavirus virus disease 2019 (COVID-19) (**Wang et al., 2020**). Reflecting its likely zoonotic origins, SARS-CoV-2-like and other SARS-related viruses have been detected and isolated from horseshoe bats and pangolins from Asia (Boni et al., 2020). SARS-CoV-2 is a highly transmissible virus with an R0 of up to ~5, and has a relatively high case mortality rate (~1%), especially pathogenic in elderly or individuals with co-morbidities (**Cevik et al., 2020**). While safe and effective vaccines were recently developed (**Krammer, 2020**), there is a dearth of highly-effective therapeutic interventions, with notable exceptions such as dexamethasone (**Recovery Group, 2021**).

SARS-CoV-2 productively infects the epithelial cells lining the upper and lower respiratory tract, including those in the nasal cavity and the alveoli of the lung (**Hou et al., 2020**). By virtue of interaction with Spike, SARS-CoV-2 exploits host cell protein angiotensin-converting enzyme 2 (ACE2) as its receptor (**Shang et al., 2020**). Additionally, for entry to occur Spike requires activation by two host proteases, furin and transmembrane protease serine 2 (TMPRSS2)-like proteases, which cleave Spike at the S1/S2 boundary between its two subunits (S1 and S2) and the S2' site in S2 allowing release of the fusion peptide (**Hoffmann et al., 2020**). Following binding to ACE2, a proteolytically-activated Spike

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can fuse the viral envelope with the host cell membrane releasing the infectious genome into the cytoplasm.

Since its initial emergence, SARS-CoV-2 has continued to evolve and adapt to the human population with several putatively beneficial mutations arising in Spike, such as D614G and N501Y, that affect Spike stability and binding to ACE2, and antibody-escape mutations in the amino-terminal domain (NTD) (**Harvey et al., 2021**). Additionally, loss of the polybasic cleavage site (PBCS), which is a unique feature of SARS-CoV-2 and facilitates furin cleavage at the S1/S2 boundary, has been demonstrated to reduce transmission and virulence of SARS-CoV-2 in animal models (**Johnson et al., 2021; Peacock et al., 2021**). Together, these mutations of interest are found in constellations in so-called 'variants of concern' (VOC), which are strains of SARS-CoV-2 with evident phenotypic differences, such as enhanced transmissibility, pathogenicity, or reduced sensitivity to antibody-mediated neutralization in humans (**Harvey et al., 2021**).

As SARS-CoV-2 continues to spread, and interventions and vaccines are being rolled out, there remain significant unknowns as to how SARS-CoV-2 may adapt further to humans. Knowledge of the genetic and molecular correlates of this difference in transmissibility is crucial for understanding of coronavirus pandemic preparedness and inform strategies for surveillance and control. In vitro models can help disentangle the factors affecting evolution, identify new ones, and highlight mutational tolerance. Here, we undertook a side-by-side comparison of SARS-CoV-2 evolution by whole genome sequencing of two isolates, grown in parallel in standard Vero-derived cells and human 'well-differentiated primary nasal epithelial cells' (WD-PNECs), which are a useful model for probing virus-host interactions in the respiratory tract (**Guo-Parke et al., 2013; Hou et al., 2020; Villenave et al., 2012**). Our data demonstrate clear roles of both viral and host cell factors in

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shaping SARS-CoV-2 genetic and functional changes, identifying genetic features required for efficient infection of primary cells.

Results

Isolation and passage of SARS-CoV-2 in unmodified Vero-derived cells

To begin to understand the evolution of SARS-CoV-2 we first needed to generate characterised stocks of virus (**Fig 1A**). In the first instance, a low passage isolate (passage 1, P1) of SARS-CoV-2 (England 02/20) was obtained from Public Health England (PHE) and is referred to as 'PHE'. This stock was from a sample isolated on VeroE6 cells and represents one of the earliest isolates of SARS-CoV-2 in the UK during the pandemic. PHE is from clade A and does not contain the D614G substitution in Spike (**supplementary table 1**)(**Davidson et al., 2020; Holden et al., 2020**). Upon receipt, we carried out a further three passages on VeroE6 cells passaging at an MOI of 0.001, harvesting stocks at 96 hpi when extensive cytopathic effect was observed. The PHE strain grew efficiently, reaching titres of $>10^6$ pfu/mL (**Fig 1B**) and was cytopathic, inducing 'webbing' and cell rounding, consistent with previous reports (data not shown).

As we wanted to understand the viral factors that may drive evolution and results obtained from only one isolate may be non-representative, we next obtained an independent – but comparable – clinical nasal/pharyngeal swab sample containing SARS-CoV-2, which we termed BT20.1 (Belfast 06/20). This strain represents an isolate from the UK's 'first wave' and is a representative of clade B that contains the D614G mutation in Spike (table 1). Unlike PHE, BT20.1 was isolated on standard Vero cells (CCL-81) and passaged to P4 (multiplicity of infection

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149 [MOI]~0.001 and passaged every 3 days). Like PHE, BT20.1 grew
150 efficiently, reaching titres of $>10^6$ pfu/mL (**Fig 1B**).

151 Both PHE and BT20.1 formed plaques on standard Vero cells in all
152 passages (**Fig 1C and D**). Comparison of plaque sizes between P2 and P4
153 identified differences in plaque size composition following Vero cell
154 passage. This was most evident for PHE, which became predominantly
155 large plaques (**Fig 1 E and F**). As observed in the plaque edge, BT20.1
156 induced consistent cell-to-cell fusion, unlike PHE (**sFig 1 A and B**).

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c.

d.

e.

f.

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Figure 1. Isolation and passage of PHE and BT20.1 in Vero-derived cells. Schematic of SARS-CoV-2 isolation/serial passage series on VeroE6 or Vero cells for PHE and BT20.1 from isolation to P4 (a). Extracellular infectivity titres for stocks generated from P2-P4 VeroE6/Vero passage for PHE and BT20.1 using plaque assay protocol on Vero cells (b). Plaque visualisation of PHE (c) and BT20.1 (d) P4, and P2 (e and f) on Vero cells. Figures were generated with the aid of Biorender (<https://biorender.com/>).

Sequencing of SARS-CoV-2 passage series in unmodified Vero-derived cells

As we had successfully generated comparable in vitro passage series for two relevant isolates of SARS-CoV-2, we next determined what genetic changes, if any, occurred during passage in unmodified Vero-derived cells. Whole genome sequences of our SARS-CoV-2 stocks at each passage were generated and minor sequence analysis (>5% minor allele frequency) was carried out, comparing variation arising to the Wuhan-Hu-1 reference (NC_045512.2) genome sequence for SARS-CoV-2 (**supplementary table 1**). Unfortunately, the sequence depth and quality were not sufficient to reconstruct a whole genome sequences for BT20.1 P1 isolate material, likely due to insufficient viral material resulting from the initial isolation. Therefore, we focused our analysis on PHE P1-4 and BT20.1 P2-P4.

Analysing mutations in the PHE passage series we identified 4 changes (C8782T; T18488T; T28144C; A29596G) relative to Wuhan-Hu-1 consistently at ~100% at all passages, likely reflecting fixation in the original virus stock (**Fig 2A**). These changes were considered intrinsic to that particular strain and were not analysed any further herein as we wished to focus on variants arising during passage. Sequencing confirmed the presence of D614 in Spike, consistent with it being an early SARS-CoV-2 isolate.

Outwith the core changes described above, two major mutations were observed: a synonymous (T23605G) and non-synonymous out-of-frame deletion (deletion of 24 nucleotides AATTCTCCTCGGCGGGCACGTAGTG 23597A; resulting in the replacement

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of of 9 amino acids [679-687; NSPRRARSV] in Spike with an isoleucine [I]) mapping to the polybasic cleavage site (PBCS) (**Fig 2A**). Deletion of the PBCS ablated the T23605G synonymous variant in the process. This occurred at P3, although the deletion was observed in the original P1 material from PHE. Furthermore, we detected 15 minor variants (non-consensus) that had an allele frequency (AF) of >5% in at least one sample of the passage series. These changes mapped to several genes and proteins of SARS-CoV-2, including ORF1AB, Spike, E, N, and ORF10. (**supplementary table 1**). Interestingly, we observed a cluster of three mutations occurring in the amino terminal domain (NTD) of Spike, appearing at P3 and rising in frequency at P4. Two of these Spike NTD mutations were similar to mutations occurring in VOCs: D215G and a deletion of 24 nucleotides (GCTATACATGTCTCTGGGACCAATGGTA21761G) resulting in a loss of 9 amino acids IHVSGTNGT (aa68-77). Additionally, we noticed a convergent mutation of L37 in E, detecting two mutations resulting in L37F and L37R. To determine the reproducibility of passage sequencing, an independent P4 PHE (P4B) was generated from P3 and sequenced, with very high levels of similarity between the two (**supplementary table 1**).

Like PHE, we identified core changes inherent to BT20.1 (**Fig 2B**), which were greater in number than PHE (10 vs 4), consistent with its later isolation (February 2020 versus June 2020) (**supplementary table 1**). These changes included, but were not limited to, D614G in Spike; R203K & G204K in N; and an out-of-frame deletion of 5 nucleotides in ORF7A leading to its premature truncation. Like PHE, we identified mutations arising rapidly upon consecutive passage in Vero cells (i.e., were not detected at P2), including the non-synonymous mutations T293I in NSP12 and P812R in Spike. Both mutations had similar patterns of change in frequency and constituted the majority of sequences by P3. Like PHE, we also detected minor variants (9), including G1251V and S1252C in Spike.

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a.

b.

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Figure 2. Analysis of PHE and BT20.1 whole genome sequences during Vero cell passage. Frequency of mutations detected for PHE (a) and BT20.1 (b) passage series on VeroE6 or Vero cells, respectively, relative to the reference sequence (Wuhan-Hu-1). Only sequences from P1-P4 (PHE) and P2-P4 (BT20.1) were analysed to facilitate adequate comparisons. Core changes are found consistently at high frequency and minor variants found at consistently low frequency (e.g. <50%). Only variants that significantly changed in frequency are marked on the graph. Colours do not reflect relationships between variants.

Passage of SARS-CoV-2 on primary human airway cultures

We next sought to investigate the effect of host cell type on subsequent viral evolution, as our previous analysis assessed the contribution of viral background to viral evolution in Vero-derived cells. To this end, in parallel, we passaged SARS-CoV-2 samples on well-differentiated primary human airway epithelial cell cultures until P4, in a similar protocol as was carried out in Vero cells (**Fig 3A**). Primary cultures included WD-PNECs derived from two paediatric donors. For both PHE and BT20.1 robust infection and passage on WD-PNECs was established. For PHE, WD-PNECs were initially infected at MOI of 0.1 and virus harvested at 2-3 dpi, using the original P1 virus material. This was repeated for BT20.1, except unlike PHE, BT20.1 was directly isolated on primary cultures from the obtained clinical material. SARS-CoV-2 grew well in the primary cultures, reaching titres of $\sim 10^6$ pfu/mL in 2-3 days in the apical compartment. Samples at each passage were subjected to sequencing as outlined above and analysed in a similar manner to those from the Vero cell passage series. For BT20.1 only P2, P3 and P4 were sequenced to compare with the data available for the equivalent Vero passage series.

In contrast to what was observed in VeroE6 cells, we did not detect any major genetic changes in PHE following passage in WD-PNECs (**Fig 3B**). However, we did identify the PBCS deletion at low levels in minor variant analysis, but never reaching majority. Together with PBCS we found 34 changes as minor variants. From passage to passage, these mutations appeared, and disappeared, stochastically. Similarly, in BT20.1, unlike the Vero cell passage, we did not find corresponding mutations in

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Nsp12 or Spike (**Fig 3C**). However, we identified a single amino acid deletion, Y1595, in NSP3. Intriguingly, this variant was maintained throughout the passage series at a moderate frequency of ~45%. At each passage, where possible, SARS-CoV-2 was titrated by plaque assay on Vero cells (**sFig 2**). However, we were unable to obtain titres for PHE and BT20.1 passage P3 and P1, respectively. We noticed slightly reduced titres of BT20.1 in primary cells at P4 compared to earlier passages, which was not observed for PHE (**sFig2**). WD-PNEC-grown viruses had less obvious plaques (**sFig 3 A and B**) and no evidence of cell-to-cell fusion was identified, even for BT20.1 (**sFig 3 C**). Similar to passage in Vero cells we identified two mutations in Spike (G1251V and S1252C), which appeared at low frequencies (<10%) and never increased (**table 1**).

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Figure 3. Analysis of PHE and BT20.1 whole genome sequences during WD-PNECs passage. Schematic of SARS-CoV-2 isolation/passage series on WD-PNECs for PHE and BT20.1 (a). Frequency of mutations detected for PHE (b) and BT20.1 (c) passage series on WD-PNECs, respectively, relative to the reference sequence (Wuhan-Hu-1). Only sequences from P1-P4 (PHE) and P2-P4 (BT20.1) were analysed. PHE P1 is the original stock material obtained and hence is the same sequence as PHE P1 in figure 2. Core changes were found consistently at high frequency and minor variants found at consistently low frequency (e.g. <50%). Only variants that significantly changed in frequency are marked on the graph. Colours do not reflect relationships between variants. Figures were generated with the aid of Biorender (<https://biorender.com/>).

Phenotypic differences between SARS-CoV-2 'PHE' and 'BT20.1' **P4**

Our data showing host cell dependency in viral evolution suggested differential fitness for specific viral genotypes (e.g., Vero cell-derived mutations that were not observed in WD-PNECs were less fit in primary cells). To test this hypothesis, we focused subsequent analysis on PHE and BT20.1 Vero P4 stocks with clear genetic differences between them, including the PHE PBCS deletion in Spike, and the P812R (Spike) and NSP12 mutations in BT20.1. To this end, we wished to directly compare the growth and multi-cycle replication kinetics of both strains in cell culture models of infection. To achieve this, we carried out a comparison of growth kinetics in several cell culture models, including Vero cells, VeroE6 cells modified to express human ACE2 and TMPRSS2 (VAT) (Rihn et al., 2021), and WD-PNECs (adult nasal) (**Fig 4A-C**). Of note, unmodified Vero and VeroE6 cells do not express human ACE2 and have very low levels of TMPRSS2 (**Matsuyama et al., 2020**). In Vero cells, SARS-CoV-2 grew to peak extracellular infectivity titres by ~48 hpi with titres of $\sim 10^6$ pfu/mL. We noticed a growth attenuation of BT20.1 in Vero cells compared to PHE (**Fig 4A**). Comparing virus growth in VAT cells (**Fig 4B**), both viruses grew better but the relative attenuation of BT20.1 was not observed in VAT cells. In contrast to previous Vero cell experiments, we observed a prominent growth defect of PHE compared to BT20.1 at early time points during infection (24/48 h) in WD-PNECs. However, both viruses reached similar titres by 72 hpi (**Fig 4C**). Together these data

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309 clearly demonstrate phenotypic differences between our Vero cell-
 310 passaged viruses, demonstrating a critical role for the PBCS for efficient
 311 replication in primary cells.

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Figure 4. Comparison of PHE P4 (Vero) and BT20.1 P4 (Vero) growth on different cell substrates. Multicycle growth curves (MOI 0.01 for Vero or 0.1 for WD-

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315 PNECs) for PHE P4 (VeroE6) and BT20.1 P4 (Vero) on Vero (a), VeroE6 cells expressing
 316 human ACE2 and human TMPRSS2 (b), and adult WD-PNECs from 3 donors (c). Titres
 317 for Vero-derived cells are shown as means +/- SEM for triplicate wells and are
 318 representative of two independent experiments. Titres for WD-PNECs are shown as
 319 means +/- SEM for single wells from 3 donors. Data using BT20.1 are presented here as
 320 averages from 3 donors but have also been incorporated into a sister paper using
 321 separated, individual donor data (Broadbent et al., 2021 in submission). Figures were
 322 generated with the aid of Biorender (<https://biorender.com/>).
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Discussion

Investigation of the patterns of SARS-CoV-2 genetic diversity worldwide during outbreaks has already facilitated a genetic-based nomenclature of lineages and has also highlighted the emergence of functionally-relevant mutations, such as D614G in Spike, and those contained in extant VOCs (**Harvey et al., 2021**). Complementary to this, in vitro systems are an incredibly useful and tractable means to understand the forces influencing this viral evolution, in particular those that mimic in vivo-relevant conditions, such as WD-PNECs (**Hou et al., 2020**).

Our data and that of others demonstrate significant standing genetic diversity in viral populations in vitro that can be acted upon by rapid evolutionary processes. Although also observed in our work, from early in the study of SARS-CoV-2 evolution in Vero-like cells, it was revealed that the virus readily diversifies during culture with the most evident being mutations mapping to the PBCS of Spike (**Davidson et al., 2020; Klimstra et al., 2020; Lamers et al., 2021; Ogando et al., 2020; Pohl et al., 2021**). Consistent with our work, other studies have identified enhanced genetic stability, in particular of the PBCS only, during passage of one strain in Calu3 or primary airway organoids (**Lamers et al., 2021**). One striking finding of our work, which builds on previous studies, is that on several occasions for both isolates we observed a rapid increase in frequency of specific mutations in the PBCS and independently of it (PBCS deletion & P812R in Spike, and T293I in NSP12) to near fixation over the course of a couple of passages in Vero cells. These patterns suggest a selective phenotypic advantage in that particular cell culture system. Similar changes (including P812R and the NTD deletion) were identified in other studies (**Dieterle et al., 2020; Ramirez et al., 2021**). The fact that identical mutations arise independently (e.g., loss of the PBCS and P812R) is highly suggestive of convergent evolution, perhaps toward a similar phenotype. Our work on the loss of the PBCS in

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Vero cells and its association with attenuation in WD-PNECs is consistent with previous reports. However, it is noteworthy that we did not observe deletion or mutations in and around the PBCS during passage of BT20.1 in Vero cells.

In addition to the loss of the PBCS, we observed P812R in Spike and T293I in NSP12, although we were not able to associate them with changes in virus growth in WD-PNECs due to a lack of an additional comparable 'wild-type' isolates. However, the fact that parallel passage in WD-PNECs did not result in their increased frequency suggests that they confer a hitherto unrecognised disadvantage in the primary epithelial cell system. P812R is a non-conservative change and rapidly rose to near-fixation alongside NSP12 in BT20.1 in Vero cells. P812 sits near the S2' cleavage site and is a highly conserved position in SARS-CoV-2. However, non-P residues (e.g. serine) were occasionally found in nature but are rare (<https://nextstrain.org/>), which suggests a functional defect in vivo. Interestingly, P812R was observed before, in at least two other studies, associated with a change in Spike activity using full-length SARS-CoV-2 and one using chimeric vesicular stomatitis virus encoding SARS-CoV-2 Spike (**Dieterle et al., 2020; Ramirez et al., 2021**). Like previous work, we noted an association of P812R with enhanced cell-to-cell fusion when BT20.1 grown on Vero cells is compared to that grown in WD-PNECs (**sFig 3**). It was suggested that P812R generated a novel PBCS at the S2' site (**Ramirez et al., 2021**). Cleavage by furin-like proteases could thus compensate for lack of TMPRSS2-mediated proteolysis and activation in Vero cells. Although it is possible that P812R confers a similar phenotypic change as the PBCS deletion, it is not likely to be identical, given the clear differences in growth between PHE and BT20.1 in Vero cells and WD-PNECs. Along with P812R in S, BT20.1 carried a mutation in NSP12 (T4685I/T293I), which is the viral RNA-dependent RNA polymerase. The mutation sits on the surface in close proximity to a zinc-binding site of the interface domain that mediates intra-NSP12 interactions and interactions

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between NSP12 and other polymerase co-factors, such as NSP8 (**Hillen et al., 2020**). Given the linkage between P812R and T4685I, further molecular virological work using isogenic viruses generated through reverse genetics is required to ascertain the impact of this mutation in relevant cell models. The mutations T4685I arose with P812R possibly suggesting genetic linkage, although this remains to be determined.

It is of interest that BT20.1 carries a deletion in ORF7A that results in a frameshift and C-terminal truncation of the protein, likely ablating the transmembrane domain and tail. ORF7A is a type 1 transmembrane protein and it has numerous putative functions involved in host-pathogen interactions and immune evasion (**Nemudryi et al., 2021**). ORF7A truncations in SARS-CoV-2 isolates have been discovered before, possibly associated with reduced capacity to subvert the innate immune response (**Nemudryi et al., 2021**). However, the previous work was carried out using non-clinically relevant cell models, such as Vero or HEK-derived lines. Our work suggests that full-length ORF7A is not required for replication in Vero or WD-PNECs and likely serves an accessory function that may affect replication and/or transmission in particular circumstances.

Not only did we observe changes reaching near-fixation in our dataset we also identified several lower frequency mutations in our viral populations. Consistent with this variation within a stock, we also noticed plaque size variation in passage stocks suggestive of functional differences between viral sub-clones (**Fig 1 E and F**). We detected an in-frame deletion of a single codon in the C-terminus of NSP3, located in the Y1 domain, which is located on the cytoplasmic face of the virus-remodelled ER membrane, where it may regulate replication complex stability by interacting with NSP4 (**Lei et al., 2018**). NSP3 itself is a multifunctional protein involved in numerous viral processes. The fact that the deletion did not rise to fixation suggests that it is at a competitive disadvantage compared to wild-type. The mutation in NSP3 is also

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interesting because it is maintained at a moderate frequency. Of considerable interest is the overlap between variations observed in Vero cells and that of VOCs, especially in the NTD of Spike. We observed three mutations in the NTD in PHE P3 and P4: E180K, D215G, and a deletion resulting in the loss of 9 amino acids. Variants identified in this study mapping to the ectodomain of Spike are marked on a structural model (**sFig 4**). For D215G and the deletion, these mutations are similar to those in VOCs, such as alpha and beta variants. Regarding mutations in NTD loops, several VOCs have convergently modified the amino acid identity of the loop. While in vivo this may be the result of antibody selection, in our system there are no antibodies, which suggests a role for NTD mutations independent of antibody selection. The rise in frequency is suggestive of a fitness advantage of these mutations. Further work is required to determine the function of the NTD of Spike and the impact of these mutations on the virus life cycle.

While general trends were similar between our two isolates in Vero cells (i.e., mutations rising to high frequency), specific mutations observed were not. It is likely that evolution of key mutations reflect inherent biological differences in viruses and not subtle changes in passaging conditions. In PHE, loss of the PBCS occurred, which was not observed in BT20.1, and vice versa regarding P812R and NSP12. This is consistent with an effect dependent on viral input or strain or genetic background through epistatic interactions between mutations, such as D614G in Spike. However, in numerous reports, isolation and passage of SARS-CoV-2 on Vero cells selected for a loss of the PBCS, which was not observed in our BT20.1 passage series. Alternatively, the mutation P812R could functionally achieve the same phenotype as the deletion of the PBCS, although our primary cell infection model where PHE was attenuated compared to BT20.1, would suggest that this is not the case.

By comparing evolution of the same isolates in two distinct cell culture systems, we observed a dependence on host cell substrate on

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downstream virus evolution. Namely, passage in WD-PNECs resulted in enhanced stability of SARS-CoV-2 genetic diversity at the consensus level. While the PBCS, P812R and NSP12 changes were identified in PHE and BT20.1 when grown in Vero cells, these changes did not rise to high frequencies in WD-PNECs. Differential accumulation of mutations may reflect distinct host cellular environments encountered upon passage in Vero or WD-PNECs. This reflects major differences in these cell substrates, including i) species and tissue differences; ii) reduced levels of TMPRSS2 in Vero cells; iii) reduced innate immune response in Vero cells as they are deficient in type 1 interferon production (**Emeny & Morgan, 1979**). However, what affects the rise in P812R/NSP12 mutation remains unknown. Future work will assess the effect of these changes in BT20.1 upon replication in WD-PNECS. Additionally, during passage of BT20.1 in WD-PNECs we identified a deletion in Nsp3, although the relevance and mechanism of this change is unknown.

In conclusion, by studying the evolution of SARS-CoV-2 during passage in distinct cellular substrates we shed light on the forces that shape viral fitness, unveiling a collaboration between both viral and host factors in driving SARS-CoV-2 genetic diversity, which helps define the molecular correlates of fitness in the natural target cells. Finally, on a practical note, our results support close characterisation of virus stocks for experimentation in vitro and in vivo and suggest ways to mitigate unwanted cell culture artefacts, critical for understanding host-pathogen interactions and identification of antiviral interventions.

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Figure legends

Figure 1. Isolation and passage of PHE and BT20.1 in Vero-derived cells. Schematic of SARS-CoV-2 isolation/serial passage series on VeroE6 or Vero cells for PHE and BT20.1 from isolation to P4 (a). Extracellular infectivity titres for stocks generated from P2-P4 VeroE6/Vero passage for PHE and BT20.1 using plaque assay protocol on Vero cells (b). Plaque visualisation of PHE (c) and BT20.1 (d) P4, and P2 (e and f) on Vero cells. Figures were generated with the aid of Biorender (<https://biorender.com/>).

Figure 2. Analysis of PHE and BT20.1 whole genome sequences during Vero cell passage. Frequency of mutations detected for PHE (a) and BT20.1 (b) passage series on VeroE6 or Vero cells, respectively, relative to the reference sequence (Wuhan-Hu-1). Only sequences from P1-P4 (PHE) and P2-P4 (BT20.1) were analysed to facilitate adequate comparisons. Core changes are found consistently at high frequency and minor variants found at consistently low frequency (e.g. <50%). Only variants that significantly changed in frequency are marked on the graph. Colours do not reflect relationships between variants.

Figure 3. Analysis of PHE and BT20.1 whole genome sequences during WD-PNECs passage. Schematic of SARS-CoV-2 isolation/passage series on WD-PNECs for PHE and BT20.1 (a). Frequency of mutations detected for PHE (b) and BT20.1 (c) passage series on WD-PNECs, respectively, relative to the reference sequence (Wuhan-Hu-1). Only sequences from P1-P4 (PHE) and P2-P4 (BT20.1) were analysed. PHE P1 is the original stock material obtained and is hence the same sequence as PHE P1 in figure 2. Core changes are found consistently at

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high frequency and minor variants found at consistently low frequency (e.g., <50%). Only variants that significantly changed in frequency are marked on the graph. Colours do not reflect relationships between variants. Figures were generated with the aid of Biorender (<https://biorender.com/>).

Figure 4. Comparison of PHE P4 (Vero) and BT20.1 P4 (Vero) growth on different cell substrates. Multicycle growth curves (MOI 0.01 for Vero or 0.1 for WD-PNECs) for PHE P4 (VeroE6) and BT20.1 P4 (Vero) on Vero (a), VeroE6 cells expressing human ACE2 and human TMPRSS2 (b), and adult WD-PNECs from 3 donors (c). Titres for Vero-derived cells are shown as means +/- SEM for triplicate wells and are representative of two independent experiments. Titres for WD-PNECs are shown as means +/- SEM for single wells from 3 donors. Data using BT20.1 have been reproduced here as averages from 3 donors but have also been incorporated into a sister paper using separated, individual donor data (Broadbent et al., 2021 in submission). Figures were generated with the aid of Biorender (<https://biorender.com/>).

Table 1. Frequency of variants in reference to Wuhan-Hu-1 identified in this study. Variants only shown where there was at least one instance of frequency >5%. Where undetectable an arbitrary value of 0.1 was assigned. Frequency data is highlighted by colour (green for higher, yellow for lower). Mutations have been assigned status of core, variant or minor. Additionally, for each variant, data for nucleotide location, reference and variant nucleotides, gene & protein location, and consequence (e.g., synonymous [S] or non-synonymous [NS]) are shown.

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sFig 1. Fusogenicity of PHE and BT20.1 P4 on Vero cells. Higher magnification images of plaque visualisation of PHE (a) and BT20.1 (b) P4 on Vero cells from the same images shown in Figure 1.

sFig 2. Growth kinetics of PHE and BT20.1 during passage in WD-PNECs. Infectivity titres for material generated from isolation/passage of PHE and BT20.1 on WD-PNECs.

sFig 3. Plaque morphology of SARS-CoV-2 grown in WD-PNECs. Plaque visualisation of PHE (a) and BT20.1 (b) P4 on Vero cells. Higher magnification images of plaque visualisation of BT20.1 (c) P4 on Vero cells from the same images shown (b).

sFig 4. Location of Spike mutant variants observed in this study on model structure of a single Spike monomer in the pre-fusion state (PDB 7C2L

from (Chi et al., 2020)). Variants identified in the Spike cytoplasmic tail (G1251V and S1252C) are not shown.

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Materials and methods

Continuous cell line culture

In this study, 3 continuous cell lines were used: Vero wildtype (number), Vero E6, and Vero E6 expressing human ACE2 and TMPRSS2 (VAT) (Rihn et al., 2021). All cells were grown in DMEM (5% FCS v/v) with antibiotics. VAT cells were maintained in the presence of additional antibiotics to select of cells carrying transgenes. Cell lines were routinely tested for mycoplasma contamination and no evidence of contamination was detected.

WD-PNECs

Nasal epithelial cells from preschool age children with recurrent wheeze (for initial passaging) and from healthy adults (for final comparison of PHE and BT20.1 P4 viruses) were obtained by brushing of the nasal turbinates with an interdental brush (DentoCare). Cells were cultured in monolayer until passage 3 then seeded onto collagen-coated Transwells (6 mm, 0.4 µm pore size; Corning). Once confluent the apical medium was removed to create an air-liquid interface which, together with specialised media (Pneumacult ALI, Stemcell Technologies), triggered differentiation (Broadbent et al. 2016. Broadbent et al. 2020). Complete differentiation (after a minimum of 21 d) was confirmed by an intact culture, extensive cilia coverage and mucus production.

Viruses

Two SARS-CoV-2 isolates were used throughout this study, including 'PHE' and 'BT20.1'. PHE was provided as an early passage isolate on VeroE6 cells while BT20.1 was provided directly as a nasopharyngeal swab in virus transport media clinical material from a positive case from

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Belfast in June 2020. Stocks were prepared in Vero or VeroE6 cells in DMEM containing 2.5% FCS (v/v) infected at a low MOI (~0.001). Infections were harvested when maximal cytopathic effect was noted, usually between 3-4 days post infection. Infected culture supernatant was harvested, clarified by centrifugation and stored at -80°C. WD-PNECs were apically infected with SARS-CoV-2 for 1 h, after which the inoculum was removed and the apical surface gently rinsed with DMEM. Virus was harvested from WD-PNECs by incubation of the apical surface with DMEM for 5 min at room temperature in the absence of serum. Harvested virus was immediately stored at -80. All SARS-CoV-2 work was carried out under BSL3 conditions in a dedicated facility in QUB.

Plaque assays

Our plaque assay protocol is based on the methodology available here: <https://www.protocols.io/view/viral-titration-of-sars-cov-2-by-plaque-assay-semi-be4zjgx6>. Near confluent monolayers of Vero cells in 24 or 6 well plates were infected. On the day of titration growth media was replaced with DMEM (0% FCS) (250 µL). Virus dilutions were prepared in plate and incubated for 30 min after which the 2x overlay medium (containing 2% agarose) was added. Plates were incubated for 3 days at 37°C. At 3 dpi PFA (8%) was added to the cultures and cells fixed/inactivated for at least 20 min. Following fixation, the PFA was removed and monolayers stained for 10 min using crystal violet (1% w/v in ethanol 20%). Following staining, residual crystal violet solution was removed, plates were rinsed in water and submerged in Chemgene prior to drying and removing from the hood for visualisation and quantification. To calculate PFU/mL, plaques at a dilution were quantified, the precipice of this number used, and multiplied by the dilution factor (4). For visualisation of plaque assays, whole plates were scanned using a Celigo imaging cytometer (Nexcelom Bioscience).

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Virus whole genome sequencing

Virus whole genome sequencing used methods developed by the ARTIC network (<https://artic.network>; (Tyson et al., 2020)) and the COG-UK Consortium. Culture supernatants were inactivated by addition of Triton X-100 to 1.5% (v/v). Viral RNA (total nucleic acid) was extracted from inactivated samples (200 µL) using the MagNA Pure Compact instrument and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Molecular Systems Inc, Burgess Hill, UK). Purified nucleic acid was eluted into 100 µL and used immediately or stored at -80°C. For first-strand cDNA synthesis, nucleic acid (5 µL) was used as template for reverse transcription using LunaScript® RT SuperMix Kit (New England Biolabs, Hitchin, UK) in 20 µL reaction volume. Primers were annealed (65°C, 5min, snap-cool on ice) prior to addition of reverse transcriptase. Reactions were incubated at 42°C (50 min) then stopped at 70°C (10 min). The resulting cDNA was used immediately for PCR or stored at -20°C. In brief, these were run as two separate multiplex PCR “pools” (A & B) using the ARTIC version 3 primer set (ARTIC nCoV-2019 V3 Panel, IDT DNA Inc, Leuven, Belgium; <https://github.com/artic-network/primer-schemes/tree/master/nCoV-2019>) and Q5 DNA polymerase mastermix (New England Biolabs). Following PCR, the amplicons from pools A & B were combined, and the resulting pooled amplicons (98 x 450 bp overlapping tiled amplicons, spanning the SARS-CoV-2 genome) were purified using Kapa HyperPure beads (Roche Molecular Systems Inc) and quantified using a Qubit fluorometer and dsDNA HS Assay Kit (Thermo Fisher Inc, Manchester, UK). Amplicon sequencing libraries were prepared using the Nextera DNA Flex library kit according to the manufacturer’s instructions (Illumina Ltd., Cambridge, UK). Libraries were sequenced on a MiSeq (Illumina) using a MiSeq Reagent Kit v2 and 2 x 151 bp paired-end sequencing protocol (Illumina).

Sequence analysis

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The FASTQ files were uploaded into the Galaxy web platform, and we used the public server at usegalaxy.eu to analyse the data (**Afgan et al., 2018**). The workflow used was specially optimized for Illumina-sequenced based ARTIC pair end data with the intention to detect allelic variants (AVs) in SARS-CoV-2 genomes (**Maier et al., 2021**). This analysis converted FASTQ data to annotated AVs through a series of steps that include QC, trimming ARTIC primer sequences off reads with the iVar package, mapping using bwa-mem, deduplication, AV calling using lofreq, and filtering AVs that both occurred at an allele frequency (AF) $\geq 5\%$, and were supported by ≥ 10 reads. As we could not determine the background frequency of mutations we focused on those variants with a minor allele frequency $\geq 5\%$, and were supported by ≥ 10 reads in at least one passage of the series. Furthermore, we focused our greater analysis on those found in more than one passage and those that substantially rise in frequency. Raw data and consensus sequences will be uploaded during review and before publishing.

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PHE VEROE6											
POS	REFERENCE	VARIANT	P1	P2	P3	P4A	P4B	CATEGORY	GENE	S/NS?	CONSEQUENCE
7749	C	T	0.1	12	21	16	19	MINOR	ORF1AB	NS	T2495I
8782	C	T	97	98	97	97	98	CORE	ORF1AB	S	
9534	C	T	0.1	0.1	2	5	5	MINOR	ORF1AB	NS	T3090I
18488	C	C	98	97	97	98	96	CORE	ORF1AB	NS	I6075T
19983	C	CT	7	6	7	9	8	MINOR	ORF1AB	NS	FRAMESHIFT, D6576*
21697	C	T	13	8	4	24	15	MINOR	ORF1AB	S	
21761	GCTATACATGCTCTGGGACCAATGGTA	G	0.1	0.1	2	10	6	MINOR	SPIKE	NS	IHVSGTNGT68-77del
21846	C	T	17	47	3	0.1	0.1	MINOR	SPIKE	NS	T95I
22100	A	A	0.1	0.1	0.1	11	15	MINOR	SPIKE	NS	E180K
22206	A	G	0.1	0.1	0.1	8	11	MINOR	SPIKE	NS	D215G
23605	T	G	97	94	0.1	0.1	0.1	VARIANT	SPIKE	S	
23597	AATTCTCTCGGGCGGCACGTAGT	A	0.6	4	89	94	94	VARIANT	SPIKE	NS	NSPRRARSV679I
25339	C	T	0.1	7	0.1	0.1	0.1	MINOR	SPIKE	S	
26353	C	T	0.1	0.1	7	16	19	MINOR	E	NS	L37F
26354	C	G	0.1	0.1	0.1	5	5	MINOR	E	NS	L37R
28144	T	C	96	98	98	97	97	CORE	ORF8	NS	L84S
28833	C	T	0.1	22	0.1	0.1	0.1	MINOR	N	NS	S187L
29366	C	T	7	34	2	0.1	0.1	MINOR	N	NS	P365S
29596	A	G	98	98	98	98	98	CORE	ORF10	NS	I13M
29637	T	C	0.1	0.1	0.1	6	5	MINOR	ORF10	NS	I27I
29844	AT	A	0.1	11	0.1	0.1	0.1	MINOR	3' UTR	S	NC
PHE WD-PNECS											
POS	REFERENCE	VARIANT	P1	P2	P3	P4		CATEGORY	GENE	S/NS?	CONSEQUENCE
241	C	T	0.1	0.1	6	0.1		MINOR	5' UTR	S	
514	TGTTATG	T	2	7	0.1	0.1		MINOR	ORF1AB	S	MV85DEL
1593	C	T	0.1	0.1	0.1	35		MINOR	ORF1AB	NS	S443F
2994	A	C	0.1	0.1	0.1	7		MINOR	ORF1AB	NS	E910A
4455	C	T	0.1	15	0.1	0.1		MINOR	ORF1AB	NS	A1397V
4928	A	T	0.1	0.1	0.1	46		MINOR	ORF1AB	NS	N1555Y
6696	C	CT	3	8	0.1			MINOR	ORF10	NS	L2146S*
6750	A	G	0.1	7	0.1	0.1		MINOR	ORF1AB	NS	N2162S
7444	A	G	0.1	0.1	10	0.1		MINOR	ORF1AB	S	
7749	C	T	0.1	7	0.1	0.1		MINOR	ORF1AB	NS	T2495I
7866	G	T	0.1	0.1	6	0.1		MINOR	ORF1AB	NS	G2534V
8782	C	T	97	98	98	98		CORE	ORF1AB	S	
9532	C	T	0.1	15	0.1	0.1		MINOR	ORF1AB	S	
11074	C	CT	1	5	0.1	0.1		MINOR	ORF1AB	NS	L3606F*
12809	C	T	0.1	0.1	0.1	5		MINOR	ORF1AB	NS	L4182F
12860	A	G	0.1	0.1	10	0.1		MINOR	ORF1AB	NS	S4199G
13604	G	A	0.1	0.1	7	0.1		MINOR	ORF1AB	NS	R4447H
16949	C	T	0.1	0.1	13	0.1		MINOR	ORF1AB	NS	P5562L
17440	C	T	0.1	35	0.1	0.1		MINOR	ORF1AB	NS	P5726S
18063	TA	T	0.1	0.1	9	0.1		MINOR	ORF1AB	NS	frameshift
18488	T	C	98	97	96	97		CORE	ORF1AB	NS	I6075T
18508	C	T	0.1	0.1	0.1	8		MINOR	ORF1AB	NS	L6082F
19983	C	CT	7	7	0.1	6		MINOR	ORF1AB	NS	FRAMESHIFT, D6576*
20178	C	T	0.1	0.1	0.1	5		MINOR	ORF1AB	S	
21697	C	T	13	0.1	8	0.1		MINOR	SPIKE	S	
21846	C	T	17	0.1	18	2		MINOR	SPIKE	NS	T95I
23277	C	T	0.1	0.1	10	0.1		MINOR	SPIKE	NS	T572I
23582	T	C	0.1	0.1	9	0.1		MINOR	SPIKE	NS	Y674H
23597	AATTCTCTCGGGCGGCACGTAGT	A	6	11	0	2		MINOR	SPIKE	NS	NSPRRARSV679I
23605	T	G	97	86	94	95		CORE	SPIKE	S	
26681	C	T	0.1	0.1	7	0.1		MINOR	M	S	
27434	C	T	0.1	0.1	6	0.1		MINOR	ORF7A	NS	T14I
27509	C	T	0.1	0.1	6	0.1		MINOR	ORF7A	NS	T39I
27814	TTG	T	0.1	0.1	10	0.1		MINOR	ORF8	NS	FRAMESHIFT
28144	T	C	96	97	96	98		CORE	ORF8	NS	L84S
28393	T	C	0.1	11	0.1	0.1		MINOR	ORF8	S	
29274	C	T	0.1	23	0.1	0.1		MINOR	N	NS	T334I
29366	C	T	7	0.1	17	0.1		MINOR	ORF10	NS	P365S
29596	A	G	98	98	98	99		CORE	10	NS	I13M
BT20.1 VERO											
POS	REFERENCE	VARIANT	P1	P2	P3	P4		CATEGORY	GENE	S/NS?	CONSEQUENCE
241	C	T	100	100	100	100		CORE	5' UTR	S	
635	C	T	7	0.1	0.1			MINOR	ORF1AB	NS	R124C
1420	C	T	98	99	99			CORE	ORF1AB	S	
1681	G	A	98	99	99			CORE	ORF1AB	S	
3037	C	T	98	99	99			CORE	ORF1AB	S	
6255	C	T	96	99	96			CORE	ORF1AB	NS	A1997V
10870	G	T	0.1	90	96			VARIANT	ORF1AB	S	
14318	C	T	0.1	85	96			VARIANT	ORF1AB	NS	T4685I
14408	C	T	98	97	98			CORE	ORF1AB	NS	P4715L
16293	CATACGT	C	0.1	0.1	7			MINOR	ORF1AB	NS	CHANGE
19983	C	CT	8	6	7			MINOR	ORF1AB	NS	FRAMESHIFT, D6576*
22311	C	T	6	0.1	0.1			MINOR	SPIKE	NS	T205I
23403	A	G	97	98	99			CORE	SPIKE	NS	D614G
23997	C	G	0.1	84	96			VARIANT	SPIKE	NS	P812R
25314	G	T	3	5	0.1			MINOR	SPIKE	NS	G1251V
25317	C	G	3	5	7			MINOR	SPIKE	NS	S1252C
25521	C	T	18	0.1	7			MINOR	ORF3A	S	
27208	C	T	8	0.1	0.1			MINOR	ORF6	NS	H3Y
27213	C	T	9	2	0.1			MINOR	ORF6	S	
27671	TTCAAG	T	100	96	96			CORE	ORF7A	NS	FRAMESHIFT and truncation
28253	C	T	19	93	99			VARIANT	ORF8	S	
28881	G	A	97	98	99			CORE	N	NS	R203K
28882	G	A	96	99	99			CORE	N	S	
28883	G	C	99	99	100			CORE	N	NS	G204R
BT20.1 WD-PNECS											
POS	REFERENCE	VARIANT	P1	P2	P3	P4		CATEGORY	GENE	S/NS?	CONSEQUENCE
241	C	T	99	100	99			CORE	5' UTR	S	
1420	C	T	99	100	97			CORE	ORF1AB	S	
1681	G	A	97	98	99			CORE	ORF1AB	S	
3037	C	T	98	99	99			CORE	ORF1AB	S	
6255	C	T	91	89	93			CORE	ORF1AB	NS	A1997V
6683	AATT	A	46	45	52			VARIANT	ORF1AB	NS	
14408	C	T	99	98	98			CORE	ORF1AB	NS	P4715L
19983	C	CT	7	6	8			MINOR	ORF1AB	NS	FRAMESHIFT, D6576
21101	G	GT	1	5	0.1			MINOR	ORF1AB	NS	FRAMESHIFT, F6948
23403	A	G	97	99	98			CORE	SPIKE	NS	D614G
25314	G	T	0.1	6	1			MINOR	SPIKE	NS	G1251V
25317	C	G	0.1	6	1			MINOR	SPIKE	NS	S1252C
27671	TTCAAG	T	100	98	100			CORE	ORF7A	NS	
28881	G	A	99	97	99			CORE	N	NS	R203K
28882	G	A	96	97	99			CORE	N	S	
28883	G	C	99	99	99			CORE	N	NS	G204R
29418	T	A	0.1	5	0.1			MINOR	N	NS	L382*

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Supplementary table 1.

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b.

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Supplementary figure 1.

Comparison of SARS-CoV-2 evolution in vitro during passage in Vero and primary human airway cells

a.

Supplementary figure 2.

Comparison of SARS-CoV-2 evolution in vitro during passage in Vero and primary human airway cells

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Supplementary figure 3.

Comparison of SARS-CoV-2 evolution in vitro during passage in Vero and primary human airway cells

Supplementary figure 4.

Comparison of SARS-CoV-2 evolution in vitro during passage in Vero and primary human airway cells

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