Clinical severe acute respiratory syndrome coronavirus 2 isolation and antiviral testing

Gregory Mathez and Valeria Cagno

Abstract
Severe acute respiratory syndrome coronavirus 2 is an RNA virus currently causing a pandemic. Due to errors during replication, mutations can occur and result in cell adaptation by the virus or in the rise of new variants. This can change the attachment receptors' usage, result in different morphology of plaques, and can affect as well antiviral development. Indeed, a molecule can be active on laboratory strains but not necessarily on circulating strains or be effective only against some viral variants. Experiments with clinical samples with limited cell adaptation should be performed to confirm the efficiency of drugs of interest. In this protocol, we present a method to culture severe acute respiratory syndrome coronavirus 2 from nasopharyngeal swabs, obtain a high viral titer while limiting cell adaptation, and assess antiviral efficiency.

Keywords
Severe acute respiratory syndrome coronavirus 2, virus isolation, antiviral assay, variant, plaque assay

Introduction
At the end of 2019, a new coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified in Wuhan. Since then, it has caused millions of deaths. Different strategies of treatment are available or under investigation such as protease inhibitor, nucleoside analogs, monoclonal antibodies, or compounds targeting the secondary structure of viral RNA. Several cell lines can be infected by this virus, which has as primary entry receptor angiotensin-converting enzyme 2 (ACE-2). Vero E6, a monkey epithelial kidney cell line, is the most used for viral culture due to the extensive cytopathic effect after infection and fast viral growth, also if it is not representative of the natural tropism of the virus.

When viruses replicate inside the host cell, mutations can occur and may be selected due to an advantage in replication in the cell line used in the laboratory. The same mutations will not occur in vivo and might cause an evolutionary divergence. Viruses can acquire the ability to bind different receptors and increase their affinity for cells that do not represent the natural tropism. For instance, these changes might affect viral entry. Cell adaptation is well known for several viruses: the use of heparan sulfate as an attachment receptor is increased after cell passaging; human immunodeficiency virus 1 (HIV-1) can adapt to have a higher fitness for infection of MT4 cells; and SARS-CoV-2 was reported as well to adapt to cell culture: increasing plaques size was observed due to a mutation in the furin-like S1/S2 cleavage site. Adaptations can be an issue during antiviral design since the antiviral activity of the drug of interest can rely on the cause or the consequences of this effect. For example, antivirals acting on the viral entry of yellow fever virus might be effective against the Asibi derived strain (17D), but not on the original strain, because they have a distinct entry route. Experiments with circulating strains with low cell passaging are therefore essential to validate antivirals' activity.

At the time of writing, the emergence of new variants is the major concern of vaccine escape and increased transmissibility of the virus. Moreover, this phenomenon is likely to increase in the future, due to the selective pressure caused by the increase of vaccine coverage and exposure of the population to natural infection. Hence, it is important to assess the antiviral efficacy of a molecule against multiple variants of interest circulating in the population, rapidly isolated from patient samples.

In this context, we established a simple pipeline to culture a clinical sample of SARS-CoV-2 for antiviral...
assays. With our protocol, it is possible to isolate SARS-CoV-2 with a minimal number of passages from a nasopharyngeal sample. We present as well methods to characterize the stock by titration, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and sequencing (Figure 1). In the end, we show how to test antiviral activity in Vero E6 cells with isolated clinical strains. Other protocols are available for the isolation of SARS-CoV-2 from different clinical samples, but the goal was to assess the reliability of qPCR and the correlation among RNA copies and infectious viruses.34–37 In opposition, our protocol achieves a better success rate from nasopharyngeal RT-qPCR positive samples and can produce viral stocks with high titer and minimal adaptations.

This protocol must be performed by personnel who received specific training for a biosafety level (BSL) 3 laboratory authorized to work with SARS-CoV-2.

**Materials and reagents**

1. 24 well plate (Costar, catalog number: 3524).
2. 6 well plate (Costar, catalog number: 3506).
3. Amphotericin B (Thermo Fisher Scientific, Gibco, catalog number: 15290018).
4. Aspirating pipet (Falcon, catalog number: 357558).
5. Avicel GP3515 (SelectChemie, DuPont, catalog number: 15290018).
6. Cell culture flask 75 cm², (Falcon, catalog number: 353135).
7. Cell spatula (TPP Techno Plastic Products AG, catalog number: 99010).
8. Cercopithecus aethiops epithelial kidney cells (Vero E6), (ATCC, catalog number: CRL-1586).
9. Crystal violet (Merck, Sigma-Aldrich, catalog number: 61135-100 g).
10. Disinfectants (bleach 13–14%, ethanol 70%).
11. dNTP Mix 10 mM each (Promega, catalog number: U1511).
12. Dulbecco’s Modified Eagle Medium (DMEM) + GlutaMAX (Thermo Fisher Scientific, Gibco, catalog number: 31966-021).
13. E.Z.NA Total RNA Kit I (Omega Bio-Tek, catalog number: R6834-02).
14. Eppendorf tubes 2 mL, (Sarstedt AG & Co, catalog number: 72.694.006).
15. Ethanol absolute (Merck, Sigma-Aldrich, catalog number: 1.00983.2500).
16. extrAXON DNA Clean-up kit (Axonlab, catalog number: 685674).

**Figure 1.** Pipeline to culture severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from the clinical swab. Vero E6 cells are infected in a 6 well plate with the transport liquid of a nasopharyngeal sample. After 3 days of infection, cells and supernatants are split into flasks. When the cells display an extensive cytopathic effect, the supernatant is harvested. Virus isolates are characterized by titration, RNA quantification, and sequencing. Created with biorender.com.
17. Fetal bovine serum (FBS), (Pan Biotech, catalog number: P40-47500).
18. Formaldehyde 37% (Merck, Sigma-Aldrich, catalog number: F1635-500 mL).
19. GoScript™ Reverse Transcriptase (Promega, catalog number: A5002).
20. GoTaq qPCR Master Mix kit (used as PCR kit) (Promega, catalog number: A6000).
21. Millex-GS Filter 0.22 µm, (Merck, Millipore, catalog number: SLGS033SS).
22. Penicillin-Streptomycin (P/S), (Merck, Sigma-Aldrich, catalog number: P4333-100 ml).
23. Personal equipment for a BSL-2 laboratory (gloves, lab coat).
24. Personal equipment for a BSL-3 laboratory (gloves, Tyvek, FFP3 mask, glasses, shoes, mobcap).
25. Phosphate buffered saline (PBS) pH 7.4 (Thermo Fisher Scientific, Gibco, catalog number: 10010-015).
26. Pipet tips (10 µL, 200 µL) (Thermo Fisher Scientific, ThermoScientific, catalog number: 2149E, 2069).
27. Pipet tips 1’000 µL, (Sorenson BioScience Inc., catalog number: 10380).
28. Primer PCR forward: 5′-TCTCTTCTTAGTAAGGTAGACTT-3′ (Spike gene).
29. Primer PCR reverse: 5′-CTAACAATAGATTCTGTTGGTTG-3′ (Spike gene).
30. Primer RT-qPCR forward: 5′-ACAGGTACGTTAATAGTTAATAGCGT-3′ (E gene).
31. Primer RT-qPCR reverse: 5′-ATATTGCAGCAGTACGCACACA-3′ (E gene).
32. Probe RT-qPCR: 5′-ACACTAGCCATCCTTACTGCGCTTCG-3′ (E gene).
33. QuantiTect Probe RT-PCR kit (Qiagen, catalog number: 204443).
34. Random primers 3 µg/mL (Thermo Fisher Scientific, Invitrogen, catalog number: 48190011).
35. RNasin Plus RNase Inhibitor (Promega, catalog number: N2611).
36. Serological pipettes (5 mL, 10 mL, 25 mL) (Falcon, catalog number: 357543, 357551, 357525).
37. Stericup Quick Release Filter 0.22 µm (Merck, Millipore, catalog number: S2GPU05RE).
38. Syringe 10 mL, (Braun, catalog number: 4616103V).
39. TRK lysis buffer (Omega Bio-Tek, catalog number: PR021).
40. Trypsin-EDTA PBS 1:250 (Animed, catalog number: 5-51F00-H).
41. Tube (50 mL, 15 mL) (Falcon, catalog number: 352070, 352096).
42. Water (Bichsel, catalog number: FE1001318).

Equipment

1. −20°C freezer.
2. −80°C freezer.
3. Biosafety hood in a BSL-2 laboratory.
4. Biosafety hood in a BSL-3 laboratory.
5. Cell counter.
6. Cell Locker (Thermo Fisher Scientific, ThermoScientific, catalog number: 50151650).
7. Centrifuge for 15–50 mL tubes refrigerated.
8. Fridge.
9. Incubator.
10. Inverted light microscope.
11. Pipet set (10 µL, 20 µL, 200 µL, 1000 µL).
12. Pipetboy.
13. RT, PCR, RT-qPCR workstation.
14. Vortex.

Procedures

In the following section, we describe the different steps to culture SARS-CoV-2 from clinical samples, characterize the newly produced virus stock (titration, RNA quantification, sequencing) (Figure 1), and test the antiviral activity of the drug of interest.

a. Culture of Vero E6. Note: the culture of Vero E6 is done under a biosafety hood.

1. Vero E6 cells are thawed in a culture flask 75 cm² in 10% FBS DMEM (see recipes).
2. Vero E6 cells are grown in an incubator at 37°C with 5% CO₂.
3. Cells are washed with PBS and detached with trypsin-EDTA when they reach 90–100% confluence.

b. Culture of clinical sample SARS-CoV-2. Note: This work needs to be done under a biosafety hood in a BSL-3 laboratory. Due to safety measures, all the infected cells are incubated and transported into a Cell Locker. To increase the success of viral isolation, clinical specimens with a low Cycle Threshold (CT) (< 25) should be used. The nasopharyngeal sample should be stored at −80°C until use and should not be frozen-thawed several times.

1. Vero E6 cells are plated in a 6 well plate (3.5*10⁵ cells/well) to reach 75% confluence after 24 h.
2. The next day, cells are infected with 100–300 µL of clinical nasopharyngeal SARS-CoV-2 sample in a total volume of 1 mL with 2.5% FBS DMEM (see recipes). Contamination by bacteria or fungi is possible. To limit the risk, the addition of antifungals or antibiotics and filtration of the sample might be beneficial.
3. Cells are incubated for 3 h at 37°C with 5% CO₂.
4. The infection mix is then removed and 2 mL of 2.5% FBS DMEM is added to the cells for 3 days in a CO₂ incubator.
5. After this period, a beginning of cytopathic effect might be observed, but the protocol can be followed as well in the absence of it. Infected cells look initially rounder and with different light refraction. At later stages, cells detach from the flask. With some isolates, it is possible to observe the formation of syncytia (i.e. fusion of neighbor cells).

6. The supernatant is collected, and the cells are washed with 1 mL of PBS and detached with 1 mL of trypsin-EDTA followed by the addition of 3 mL of 10% FBS DMEM to neutralize the trypsin effect. Two milliliters of uninfected Vero E6 cells and detached infected Vero E6 cells are added in a culture flask 75 cm² with 1 mL of supernatant collected and fresh media at 2.5% FBS to reach the final volume of 12 mL.

7. Vero E6 cells are incubated at 37°C in a CO₂ incubator until they display extensive cytopathic effect (generally after 4–5 days).

8. Supernatant and scraped cells are collected and vortexed. Cells and debris are removed by centrifugation at 2*10³ r/min for 5 min. The supernatant is aliquoted and stored at −80°C. The aliquot should be frozen-thawed only once.

c. Characterization of the isolate

c.1 Titration

1. Vero E6 cells are plated in a 24 well plate (10⁵ cells/well) to reach 100% confluence after 24 h.

2. Cells are infected in duplicates with serial dilutions of viruses (e.g. 10⁻³–10⁻⁷) in 2.5% FBS DMEM with a total volume of 200 µL/well for 1 h at 37°C. A non-infected control is added for each titration.

3. The infection mix is removed and 500 µL of 0.4% Avicel GP3515 in 2.5% FBS DMEM is added in each well.

4. Cells are incubated for 2 days at 37°C with 5% CO₂.

5. The supernatant is removed and 4% formaldehyde (see recipes) is added to the wells (500 µL) for 20 min at room temperature.

6. Formaldehyde is removed and 500 µL of Crystal violet (see recipes) is added for 20 min to stain the wells.

7. The wells are washed once with water and dried.

8. The number of plaques in each well is counted under an inverted light microscope (magnification 4×) (Figure 2).

9. The titer of virus stock in plaque-forming unit (PFU)/mL is the average of individual titration for each dilution:

\[
\text{Titer (PFU/mL)} = \frac{\sum \text{plaques}}{\text{number of replicates}} \times \text{dilution factor} \\
\times \frac{1}{\text{volume of infection (0.25)}},
\]

The numbers in parentheses represent what was described in the protocol in step c.1.2.

c.2 Quantification of RNA copies

1. Fifty microliters of virus stock are lysed with 300 µL of TRK lysis buffer.

2. Extraction of total RNA is done as described by the fabricant Omega Bio-Tek.

3. RT-qPCR is performed according to the protocol of Corman et al.³⁸

c.3 Sequencing

1. For a small region of interest, RNA is extracted and reverse transcription is performed according to the protocol of the manufacturer. Afterward, a PCR is performed with the desired primers. To identify mutations on the spike gene, the protocol of the University Hospital of Geneva can be followed. After the PCR clean-up column, the amplification product can be subjected to Sanger sequencing.

2. For complete genome sequencing, we recommend to follow the method of Kubik et al.⁴⁰

d. Antiviral activity. Note: We do not describe the assessment of toxicity of the compounds since it is not the main goal of our protocol and we assume that the antiviral evaluation is performed at non-toxic doses. Moreover, any effect of the solvent on cell viability or the virus should be excluded. In the following section, we used a 24 well plate for our antiviral assays since we are able to count plaques under the microscope in 48 h. Other sizes of a plate can be used, that is, 6 well plate and longer incubation times to have larger plaques countable by eye, or smaller formats in which perform immunostaining to detect infection as described by others.⁴¹,⁴²
1. One hundred thousand Vero E6 cells per well are plated in a 24 well plate.
2. The next day, cells are infected with 100 PFU/well in 2.5% FBS DMEM for 1 h at 37°C.
3. Cells are treated with 500 µL of serial dilutions of the drug, at non-toxic concentrations, in 0.4% Avicel diluted in 2.5% FBS DMEM.
4. Vero E6 cells are incubated for 2 days in a CO2 incubator.
5. Cells are fixed with 4% formaldehyde and stained with crystal violet as previously described.
6. Plaques of SARS-CoV-2 are counted (Figure 3).
7. Percentages of infection are calculated by comparing the number of plaques in the treated wells with the wells containing equal volumes of solvent. The half-maximal inhibitory concentration (IC50) is calculated with Prism 8 (GraphPad, San Diego, US).

Expected results

After isolation of different clinical isolates as described above, examples of titers obtained for antiviral assays and mutation that occurred during the isolation are shown in Table 1. Sixteen clinical samples out of 18 were isolated with this protocol. The two non-isolated samples had high initial CT and low starting material. The percentage of samples without any mutation after the isolation represents 54%. Samples with at least two mutations represent 15%.

An example of dose–response antiviral activity against SARS-CoV-2 is shown in Figure 4. The results were assessed by plaque assay, as described in this protocol, using merafloraxcin that was previously reported to inhibit SARS-CoV-2 by interacting with its viral RNA.7 The data are presented as a percentage of infection calculated in comparison to untreated cells. The dose axis is represented in a logarithmic scale. IC50 can be calculated with GraphPad Prism software (nonlinear regression -> log(inhibitor) vs. response (four parameters) -> bottom and top constraints are 0, 100, respectively). \( R^2 \) values and confidence interval have to be critically observed. IC50s with \( R^2 \) values < 0.5 and a large confidence interval represent high variability between the replicates and require additional experiments. IC50s greater than the higher dose tested should be

| Table 1. Lineage, titer and mutation of different strains after isolation using this protocol. |
|-----------------|-----------------|-----------------|
| SARS-CoV-2 lineage | Titer (PFU/mL) | Mutation         |
| B.1.160          | 1.78 * 10^5     | None            |
| B.1.177          | 2.46 * 10^6     | NS328L          |
| B.1.238          | 2.09 * 10^6     | None            |
Drugs with low IC$_{50}$ and high selectivity index (ratio between the half-maximal cytotoxic concentration (CC$_{50}$) and the IC$_{50}$) (e.g. > 10) warrant further investigation.

**Conclusion**

SARS-CoV-2 caused an important pandemic and treatments are still under investigation. Since the adaptation of viruses to cell culture can bias the results, and viral variants are emerging with high frequency, drug efficiency should be confirmed with circulating strains. However, material from a nasopharyngeal sample is insufficient for characterization and antiviral testing.

Here, we presented a simple way to culture clinical SARS-CoV-2 without extensive passaging. Our method allowed us to isolate clinical strains from nasopharyngeal samples and limit cell adaptation. Our strategy can be applied as well to other viruses. The limitation of our protocol is that cell adaptation cannot be fully omitted since a balance between fast viral growth and cytopathic effect evaluation obliged us to use Vero E6 cells. These cells are not the natural host cell for SARS-CoV-2. Therefore, confirmation of the antiviral activity should be performed in a representative model such as human lung adenocarcinoma cell line (Calu-3) or respiratory tissues. This protocol was used to isolate different SARS-CoV-2 variants from patients for quality control of sequencing and antiviral assays for manuscripts in preparation.

**Recipes**

10% FBS DMEM
- 500 mL DMEM.
- 50 mL FBS (It has been previously decomplemented for 30 min at 56°C and filtered with a 0.22 µm filter).
- 5 mL P/S.

2.5% FBS DMEM
- 500 mL DMEM.
- 12.5 mL FBS decomplemented and filtered.
- 5 mL P/S.

2.4% Avicel
- 7.2 g Avicel.
- 300 mL water.

Dissolve with a magnetic bar for 10 min and autoclave.

Crystal violet solution
- 0.5 g crystal violet.
- 100 mL ethanol absolute.
- 400 mL water.

4% Formaldehyde
- 108.1 mL formaldehyde 37%.
- 891.9 mL PBS.
Troubleshooting

| Step | Problem | Possible reason | Solution |
|------|---------|----------------|----------|
| b6   | Contamination with bacteria and/or fungi | The nasopharyngeal sample contained bacteria and/or fungi | Repeat infection of the 6 well plate with filtered original material or in presence of antifungal/antibiotics. |
| b8   | A low cytopathic effect observed | The concentration of the virus is low | A new start in the 6 well plate can be performed with a longer incubation time or a higher amount of starting material. An additional passage with new Vero E6 cells and 6 mL of supernatant of the previous flask can be done. However, the additional passage may cause an increase in mutations |
| b8   | Contamination with bacteria and/or fungi | Viruses are not enough diluted | Wells with a high number of plaques should not be counted. Repeat titration starting with a higher dilution. |
| c.1.8 | A high number of plaques | Viruses are too much diluted | Repeat titration by starting with a lower dilution If it concerns a large portion of the well, repeat the experiment by taking care leaving the cells without medium for the shortest possible time |
| c.1.8/ d.1.6 | A low number of plaques | The cells are dried | Increase incubation time up to 3 days with an overlay of 0.6% Avicel. Another possibility is to decrease the amount of Avicel or repeat the experiment with less confluent cells. |
| c.1.8/ d.1.6 | Holes without cells that do not correspond to a plaque and are | Overconfluent cells at the time of infection | | |
| c.1.8/ d.1.6 | Plaques are too small to count | | |
| d.1.6 | A high number of plaques | | |
| d.1.6 | A low number of plaques | | |
| d.1.6 | | Overestimated titration | Repeat the experiment with fewer viruses during infection or titer again the virus stock. |
| c.1.8/ d.1.6 | | Underestimated titration | Repeat the experiment with more viruses during infection or titer again the virus stock. |

Declaration of conflicting interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
This research was funded by SNSF, grant PZ00P3_193289 to V.C.

ORCID iDs
Gregory Mathez https://orcid.org/0000-0002-4453-7649
Valeria Cagno https://orcid.org/0000-0002-5597-334X

References
1. Zhou P, Yang X-L, Wang X-G, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 2020; 579: 270–273.
2. COVID Live Update: 179,698,451 Cases and 3,891,365 Deaths from the Coronavirus - Worldometer, https://www.worldometers.info/coronavirus/ (accessed 22 June 2021).
3. Valle C, Martin B, Touret F, et al. Drugs against SARS-CoV-2: What do we know about their mode of action? Rev Med Virol 2020; 30: 1–10.
4. Campos DdO, Fulco UL, de Oliveira CBS, et al. SARS-CoV-2 virus infection: Targets and antiviral pharmacological strategies. J Evid Based Med 2020; 13: 255–260.
5. Gathiram P, Moodley J and Khaliq OP. Covid-19 pandemic: Perspectives on management. J Reprod Immunol 2021; 146: 103344.
6. Taylor PC, Adams AC, Hufford MM, et al. Neutralizing monoclonal antibodies for treatment of COVID-19. Nat Rev Immunol 2021; 21: 382–393.
7. Bhatt PR, Scaiola A, Loughran G, et al. Structural basis of ribosomal frameshifting during translation of the SARS-CoV-2 RNA genome. Science 2021; 372: 1306–1313.
8. Cagno V. SARS-CoV-2 cellular tropism. The Lancet Microbe 2020; 1: e2–e3.
9. Chu H, Chan JF-W, Yuen TT-T, et al. Comparative tropism, replication kinetics, and cell damage profiling of SARS-CoV-2 and SARS-CoV with implications for clinical manifestations, transmissibility, and laboratory studies of COVID-19: an observational study. Lancet Microbe 2020; 1: e14–e23.
10. Mathez G and Cagno V. Viruses like sugars: how to assess glycan involvement in viral attachment. Microorganisms 2021; 9: 1238.
11. Ogando NS, Dalebout TJ, Zevenhoven-Dobbe JC, et al. SARS-coronavirus-2 replication in Vero E6 cells: replication kinetics, rapid adaptation and cytopathology. J Gen Virol 2020; 101: 925–940.
12. Cagno V, Tseligka ED, Jones ST, et al. Heparan sulfate proteoglycans and viral attachment: true receptors or adaptation
bias? Viruses 11. DOI: 10.3390/v11070596. Epub ahead of print 1 July 2019.
13. Sa-Carvalho D, Rieder E, Baxi B, et al. Tissue culture adaptation of foot-and-mouth disease virus selects viruses that bind to heparin and are attenuated in cattle. J Virol 1997; 71: 5115–5123.
14. Bernard KA, Klimstra WB and Johnston RE. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. Virology 2000; 276: 93–103.
15. Klimstra WB, Ryman KD and Johnston RE. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. J Virol 1998; 72: 7357–7366.
16. Smit JM, Waarts B-L, Kimata K, et al. Adaptation of alphaviruses to heparan sulfate: interaction of Sindbis and Semliki forest viruses with liposomes containing lipid-conjugated heparin. J Virol 2002; 76: 10128–10137.
17. Bochkov YA, Watters K, Basnet S, et al. Mutations in VP1 and 3A proteins improve binding and replication of rhinovirus C15 in HeLa-E8 cells. Virology 2016; 499: 350–360.
18. Khan AG, Pickl-Herk A, Gajdzik L, et al. Entry of a heparan sulphate-biding HRV8 variant strictly depends on dynamin but not on clathrin, caveolin, and flotillin. Virology 2011; 412: 55–67.
19. Vlasak M, Goessler I and Blaa D. Human rhinovirus type 89 variants use heparan sulfate proteoglycan for cell attachment. J Virol 2005; 79: 5963–5970.
20. Wang Y and Pfeiffer JK. Emergence of a large-plaque variant in mice infected with coxsackievirus B3. mBio 2016; 7: e00119.
21. Fernandez-Garcia MD, Meertens L, Chazal M, et al. Vaccine and wild-type strains of yellow fever virus engage distinct entry mechanisms and differentially stimulate antiviral immune responses. mBio 2016; 7: e01956–01915.
22. Liu H, Chiu S-S and Chen W-J. Differential binding efficiency between the envelope protein of Japanese encephalitis virus variants and heparan sulfate on the cell surface. J Med Virol 2004; 72: 618–624.
23. Lee E, Hall RA and Lobigs M. Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. J Virol 2004; 78: 8271–8280.
24. Lee E and Lobigs M. Mechanism of virulence attenuation of glycosaminoglycan-binding variants of Japanese encephalitis virus and Murray Valley encephalitis virus. J Virol 2002; 76: 4901–4911.
25. Mandl CW, Kroschewski H, Allison SL, et al. Adaptation of tick-borne encephalitis virus to BHK-21 cells results in the formation of multiple heparan sulfate binding sites in the envelope protein and attenuation in vivo. J Virol 2001; 75: 5627–5637.
26. de Haan CAM, Haijema BJ, Schellen P, et al. Cleavage of group 1 coronavirus spike proteins: how furin cleavage is traded off against heparan sulfate binding upon cell culture adaptation. J Virol 2008; 82: 6078–6083.
27. Silva LA, Khomandiak S, Ashbrook AW, et al. A single-amino-acid polymorphism in chikungunya virus E2 glycoprotein influences glycosaminoglycan utilization. J Virol 2014; 88: 2385–2397.
28. Gardiner CL, Hritz J, Sun C, et al. Deliberate attenuation of chikungunya virus by adaptation to heparan sulfate-dependent infectivity: a model for rational arboviral vaccine design. PLoS Negl Trop Dis 2014; 8: e2719.
29. van Opijnen T, de Ronde A, Boerlijst MC, et al. Adaptation of HIV-1 depends on the host-cell environment. PLoS One 2007; 2: e271.
30. Garcia-Beltran WF, Lam EC, Denis KS, et al. Multiple SARS-CoV-2 variants escape neutralization by vaccine-induced humoral immunity. Cell 2021; 184: 2372–2383. e9.
31. Erol A. Are the emerging SARS-COV-2 mutations friend or foe? Immunit Lett 2021; 230: 63–64.
32. Aleem A, Akbar Samad AB and Slenker AK. Emerging Variants of SARS-CoV-2 And Novel Therapeutics Against Coronavirus (COVID-19). In: StatPearls. Treasure Island (FL): StatPearls Publishing, 2021. http://www.ncbi.nlm.nih.gov/books/NBK570580/ (accessed 21 June 2021).
33. Harvey WT, Carabelli AM, Jackson B, et al. SARS-CoV-2 variants, spike mutations and immune escape. Nat Rev Microbiol 2021; 19: 409–424.
34. Huang C-G, Lee K-M, Hsiao M-J, et al. Culture-based virus isolation to evaluate potential infectivity of clinical specimens tested for COVID-19. J Clin Microbiol 2020; 58: e01068–20.
35. L’Huillier AG, Torriani G, Pigny F, et al. Culture-competent SARS-CoV-2 in nasopharynx of symptomatic neonates, children, and adolescents. Emerg Infect Dis 2020; 26: 2494–2497.
36. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. Nature 2020; 581: 465–469.
37. Marot S, Calvez V, Louet M, et al. Interpretation of SARS-CoV-2 replication according to RT-PCR crossing threshold value. Clinical Microbiology and Infection 0. Epub ahead of print 29 January 2021. DOI: 10.1016/j.cmi.2021.01.017.
38. Corman V, Bleicker T, Brünink S, et al. Diagnostic detection of 2019-nCoV by real-time RT-PCR. 2020; 13.
39. Hôpitaux Universitaires Genève. Protocol for specific RT-PCRs for marker regions of the Spike region indicative of the UK SARS-CoV-2 variant B.1.1.7 and the South African variant 501Y.V2, https://www.hug.ch/sites/interhug/files/structures/laboratoire_de_virologie/protocol_ amplification_voc_20201201_uk_geneva.pdf (2020, accessed 21 June 2020).
40. Kubik S, Marques AC, Xing X, et al. Recommendations for accurate genotyping of SARS-CoV-2 using amplicon-based sequencing of clinical samples. Clin Microbiol Infect. DOI: 10.1016/j.cmi.2021.03.029. Epub ahead of print 2 April 2021.
41. Mirabeli C, Wotring JW, Zhang CJ, et al. Morphological cell profiling of SARS-CoV-2 infection identifies drug repurposing candidates for COVID-19. Proc Natl Acad Sci U S A 2021; 118: e2105815118.
42. Holwerda M, V’kovski P, Wider M, et al. Identification of an antiviral compound from the pandemic response box that efficiently inhibits SARS-CoV-2 infection in vitro. Microorganisms 2020; 8: E1872.
43. Gasbarri M, V’kovski P, Torriani G, et al. SARS-CoV-2 inhibition by sulfonated compounds. *Microorganisms* 2020; 8: E1894.

44. Cagno V, Magliocco G, Tapparel C, et al. The tyrosine kinase inhibitor nilotinib inhibits SARS-CoV-2 in vitro. *Basic Clin Pharmacol Toxicol* 2021; 128: 621–624.

45. Kiani P, Scholey A, Dahl TA, et al. In vitro assessment of the antiviral activity of ketotifen, indomethacin and naproxen, alone and in combination, against SARS-CoV-2. *Viruses* 2021; 13: 558.

46. Hahn F, Wangen C, Häge S, et al. IMU-838, a developmental DHODH inhibitor in phase II for autoimmune disease, shows anti-SARS-CoV-2 and broad-spectrum antiviral efficacy in vitro. *Viruses* 2020; 12: 1394.

47. Choy K-T, Wong AY-L, Kaewpreedee P, et al. Remdesivir, lopinavir, emetine, and homoharringtonine inhibit SARS-CoV-2 replication in vitro. *Antiviral Res* 2020; 178: 104786.

48. Abbott TR, Dhamdhere G, Liu Y, et al. Development of CRISPR as an antiviral strategy to combat SARS-CoV-2 and influenza. *Cell* 2020; 181: 865–876.e12.

49. Hoffmann M, Mösbauer K, Hofmann-Winkler H, et al. Chloroquine does not inhibit infection of human lung cells with SARS-CoV-2. *Nature* 2020; 585: 588–590.

50. Essaidi-Laziosi M, Brito F, Benaoudia S, et al. Propagation of respiratory viruses in human airway epithelia reveals persistent virus-specific signatures. *J Allergy Clin Immunol* 2018; 141: 2074–2084.