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FOCUS ON SARS-COV-2

Virus isolation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) for diagnostic and research purposes

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Summary

Isolation of the new pandemic virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is essential for diagnostic and research purposes including assessment of novel therapeutics. Several primary and continuous cell lines are currently used, and new organoid and engineered cell lines are being developed for improved investigation and understanding of the human immune response to this virus. Here we review the growth of SARS-CoV-2 in reference standard cell lines, engineered cell lines and new developments in this field.

Key words: Severe acute respiratory syndrome coronavirus 2; SARS-CoV-2; virus culture; cell culture; respiratory virus.

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INTRODUCTION

Virus culture has been regarded as the reference standard of diagnostics for decades, as it allows for identification and isolation of active, replicating virus.[1] However, more rapid and sensitive molecular techniques, typically nucleic acid amplification tests (NAAT), such as real-time polymerase chain reaction (PCR), are now the major routine diagnostic tests used in virology diagnostic laboratories. Particularly with a novel or emerging virus such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), there are certain circumstances where virus isolation for diagnostic and research purposes remains important, including:

1. To test convalescent sera for neutralising potential, for example as therapeutics for coronavirus disease 2019 (COVID-19) patients in intensive care units.
2. To determine whether infectious virus is present, particularly to inform return to work for previously infectious individuals such as health care workers; individuals with persistent PCR positive results on serial follow-up samples for viral clearance purpose; or when to discontinue transmission-based precautions for patients.[2]
3. As first line testing for SARS-CoV-2 inactivation efficacy of potential preventative or therapeutic compounds.
4. For use as positive controls in the evaluation of molecular assays.[3]

The first culture of SARS-CoV-2 internationally was reported by Caly and colleagues in Melbourne, Australia on 28 January 2020 and the cultured virus was rapidly shared globally with other researchers.[4] The World Health Organization declared the novel coronavirus a virus of Public Health Emergency of International Concern shortly afterwards on 30 January, and a pandemic on 11 March 2020.

Initial studies of SARS-CoV-2 virus culture were performed using the monkey kidney cell lines Vero-CCL81 and Vero E6.[4,5] However, various cell lines have been reported as able to sustain SARS-CoV-2 growth and offer a closer approximation to the human immune response. We review here the growth of the virus in existing standard (monkey and human) and engineered cell lines. We discuss the utility of human cell lines in virus culture, and the potential for using these in human immunological and other studies.

SARS-CoV-2 VIRUS ISOLATION

The SARS-CoV-2 virus requires the angiotensin converting enzyme 2 (ACE2) receptor[6] for entry into the host cell. This receptor is expressed in lung epithelial cells as well as endothelial cells lining the arteries, veins, capillaries, small intestine, testes, renal tissue and cardiovascular tissue.[7–9] Infection of the host cell also relies on priming of the SARS-CoV-2 spike protein by the transmembrane serine protease (TMPRSS).[6] The ACE2 receptor is also used as the receptor for both SARS-CoV and the related human respiratory alphacoronavirus NL63.[8]

Clinical samples being collected for SARS-Cov-2 nucleic acid amplification tests are upper respiratory tract samples, typically sampling both the nose/nasopharyngeal and throat (oropharyngeal) with (preferably) flocked swabs, as recommended by the Australian Public Health Laboratory Network[10]

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and World Health Organization.[11] Lower respiratory tract samples including sputum (if produced) and bronchoalveolar lavage are collected if the lower respiratory tract is suspected to be involved, although risk of virus aerosolisation is higher.[11] The virus is detectable by reverse transcription quantitative PCR (RT-qPCR) in the stool of ~30% patients, and while this may not be infectious, SARS-CoV-2 in one stool sample from a Chinese patient who died from COVID-19 was reported to be culturable after second round passage.[12] The virus is not commonly detected in urine; only one of nine patients had a very low level of SARS-CoV-2 in urine detected by real-time PCR in a study by Peng and colleagues.[13]

Non-human cell lines: Vero and Vero E6 cells

Culture of SARS-CoV-2 must take place in certified Physical Containment Level 3 (PC3) Laboratories. For routine virus culture or virus expansion, Vero CCL-81 or Vero E6 cell lines are commonly used which contain an abundance of ACE2 receptors. The virus is cultured in a CO_2-regulated tissue culture incubator at 37°C in viral culture media (Minimum Essential Media with 2% fetal bovine serum and final concentrations of penicillin 100 units/mL, streptomycin 100 μg/mL and L-glutamine 2 mM). The culture is checked for cytopathic effect (CPE) daily, which should be obvious 3 days after infection.[5] The majority of SARS-CoV-2 virus culture globally is being performed in Vero E6 cells,[14] and this cell line provides a good basis for research studies screening potential therapeutic and antiviral compounds.[15] Several other non-human cell lines including *Rhinolophus sinicus* (bat) lung cells, Rhesus monkey kidney cells and Madin–Darby canine kidney cells have been used to infect SARS-CoV-2 with varying success rates but are not used for routine culture purposes.[16]

Ogando and colleagues have reported that SARS-CoV-2 exhibits unusual phenotypic variation within two-three passages in Vero E6 cells.[15] The virus appears to evolve rapidly when passaged in Vero E6 cells, particularly in the spike protein furin cleavage region (Arg682 to Gln mutation). The mutation of the cleavage region may reduce the dependence of SARS-CoV-2 on furin, allowing it to replicate faster in Vero E6 cells.[15]

Some respiratory viruses including influenza demonstrate more efficient propagation when passaged in the presence of trypsin in the culture media.[17] Early virus isolation of SARS-CoV-2 was performed in the presence of 4 μg/mL trypsin[4]; however, subsequent groups, including our own, have passaged the virus in the absence of trypsin to high viral titres.[5]

Infectious titres of SARS-CoV-2 in Vero E6 are quantified by endpoint titration and plaque assay. For endpoint titration, serial 10-fold dilutions of cultured SARS-CoV-2 stock are prepared across a 96-well plate using viral culture media. The dilutions are then used to inoculate a seeded 96-well plate, or cells can be added to the dilutions in suspension. Plates are stored in a humidified tissue culture incubator at 37°C (5% CO_2) for 3 days, and then observed for CPE. The 50% tissue culture infectious dose ($TCID_{50}$) is commonly calculated by either the Reed–Muench method[18] or Spearman–Karber method.[19,20] To determine viral titre by plaque assays, a confluent monolayer of cells is infected with serially diluted SARS-CoV-2 of unknown concentration. Following infection, wells are overlayed with an immobilising media such as carboxymethyl cellulose (CMC), that restricts viral propagation to neighbouring cells, leading to the formation of plaques. Plaques are countable under a standard brightfield microscope, though often cellular monolayers are fixed and counterstained to make plaques visible to the naked eye. The plaque count is used to calculate viral titre in plaque forming units per millilitre (PFU/mL).[21]

Confirmation of SARS-CoV-2 growth can be performed using specific in-house[22] or commercially available real-time PCR assays to assess viral replication. Many commercial assays have recently become available, including the Allplex SARS-CoV-2 Assay (Seegene, Korea), which targets the N, and RdRP/S genes of SARS-CoV-2, and the E gene of *Sarbecovirus* (Betacoronavirus subgenus B). Many of these new SARS-CoV-2 molecular assays are being evaluated by the Foundation for Innovative New Diagnostics (FIND), according to criteria including limit of detection, regulatory status and availability of the producing company's other products in low and middle-income countries, and results will be available on the FIND website in the coming months.[23]

In addition to nucleic acid assays, growth of SARS-CoV-2 in culture can also be confirmed by immunostaining techniques, which has the advantage of being able to resolve infection patterns at the single-cell level and/or within tissue sub-architecture. Immunostaining requires antibodies or other immunoreagents which can be: (1) naturally specific against SARS-CoV-2 immunogens (e.g., sera from convalescent patients or experimentally challenged animal models, (2) engineered for SARS-CoV-2 specificity, or (3) display significant cross-reactivity against the novel pathogen. Indeed, antibody cross-reactivity within the group of beta coronaviruses has been reported in multiple studies, ranging from partial to extensive.[24] Numerous SARS-CoV antibodies (including examples against all major structural proteins) have been shown to also bind the corresponding SARS-CoV-2 antigens, including the relatively genetically diverse Spike.[15,25–27] This allows repurposing of these antibodies for several applications including immunofluorescence, Western blot and enzyme-linked immunosorbent assay (ELISA).[25]

Human cell lines

Human continuous cell lines that support the growth of SARS-CoV-2 include Calu3 (pulmonary cell line) and Caco2 (intestinal cell line).[16] More modest growth is seen in Huh7 (hepatic cell line),[15] 293T (renal cell line) and U251 (neuronal cell line).[16] Human continuous cell lines that do not support the growth of SARS-CoV-2 include A549 (lung epithelial cells), HeLa (cervical cells) and RD (rhabdomyosarcoma muscle cells),[16] which could potentially be explained by lack of ACE2 expression in these cell lines as reported by Nie *et al.*[28]

Primary cell cultures can be developed directly from human tissue, theoretically resulting in a closer approximation to *in vivo* virus growth. Human primary nasal or bronchial cell models are not currently suitable for diagnostic purposes due to challenges and expenses required for maintenance, as well as difficulties in propagation. However, they are useful for research purposes, particularly in developing individual patient-derived organoids for investigation of interesting and diverse immune responses.[29] New culture systems including air-liquid interface, nose and lung organoids are possibilities for SARS-CoV-2 research in understanding the human immune response to this virus, which in

some patients causes severe complications of lungs, liver, kidney and/or heart.[30,31]

Engineered cell lines

Cell lines can be genetically engineered to express proteins that assist in the entry of SARS-CoV-2, potentially producing higher viral loads and allowing for entry of the virus into previously impermissive cells. For example, Vero E6 cells can be engineered to express TMPRSS2, which are reported by Matsuyama and colleagues to be superior to human lung tissue and Calu-3 cells for SARS-CoV-2 culture.[32] In addition, several groups have engineered human cell lines such as 293T, HT1080, BHK-1 and HeLa to express ACE2 and these cells have been used to examine SARS-CoV-2 receptor usage, virus entry pathways and antiviral activity of SARS-CoV-2 antibodies.[6,33–38] Whilst these cell lines have been largely used in pseudoparticle based neutralisation assays, a more recent study by Rogers *et al.* used ACE2-HeLa cells to do a full virus neutralisation assay with convalescent donors. In this study HeLa-ACE2 cells were shown to be more sensitive at detecting replicating virus than the commonly used Vero E6 cells.[39]

Overexpression of TMPRSS2a in ACE2-293T can make these cells more permissive to SARS-CoV-2 infection. Virus entry in ACE2-293T occurs primarily through endocytosis, even when the viral spike protein is in its cleaved state[35] and has been shown to be cathepsin B/L dependent.[6] Co-expression of TMPRSS2 not only increases entry efficiency by juxtaposing ACE2 and TMPRSS2a in close vicinity, but likely additionally enhances virus replication by cleaving S proteins on exiting virions and by promoting syncytium formation.[40] Future studies could examine the feasibility of using these highly permissive cells in SARS-CoV-2 culture to generate high titre virus stocks, in diagnosis of samples with low viral loads and identification of virus strains with low receptor affinities. Other studies may test if these cells can serve as a more sensitive target in neutralisation assays with live replicating virus than Vero E6 cells.

NON-CULTURE BASED METHODS TO DETECT NEUTRALISING ANTIBODIES

As the pandemic spreads and vaccines are developed, it is critical to have laboratory tools available to measure serological response following vaccination or natural SARS-CoV-2 infection, as well as potential for using convalescent plasma and monoclonal antibodies as therapeutics. The current reference standard for detecting neutralising antibodies is the virus neuralisation test (VNT), requiring live SARS-CoV-2 virus. Currently SARS-CoV-2 viral isolation is being done in facilities that are PC3 and above, consistent with recommendations for SARS-CoV, pending specific SARS-CoV-2 international recommendations.[41] In general, serial dilutions of serum are incubated for 1 hour at 37°C with an equal volume of 200 $TCID_{50}$ SARS-CoV2 isolate. The serum-virus mixture is then added to Vero E6/other cell monolayers or to cells in suspension in tissue culture plates and incubated at 37°C. After 3 days, the plates are observed for cytopathic effect, and neutralisation titre of the antibody is determined by calculating the proportion of replicates protected from infection. This method requires a high degree of technical skill and has a long turnaround time not suitable for high-throughput, rapid diagnosis.

A number of research groups have reported new developments using purified proteins, pseudotyped or chimaeric viruses, which offer a rapid convenient way to measure neutralising antibody levels outside of specialised PC3 facilities. Tan and colleagues have recently published a potential alternative to live virus neutralisation [SARS-CoV-2 surrogate virus neutralisation test (sVNT)] which can be carried out in a PC2 level facility.[42] They coated enzyme-linked immunosorbent assay (ELISA) plates with ACE2 receptor protein, and then added convalescent patient sera and the receptor binding domain (RBD) of the SARS-CoV-2 spike protein conjugated to horseradish peroxidase (HRP). When virus neutralising antibodies were present in the test sera, the RBD-ACE2 binding was blocked in a dose-dependent manner, indicated by a quantifiable reduction in colorimetric signal after addition of a chromogenic substrate. Tan *et al.* report that this method had 99.93% specificity and 95–100% sensitivity when tested against two cohorts of convalescent plasma, and showed strong correlation with the conventional virus neutralisation assay (R^2=0.8591, p<0.0001).[42]

Lentiviruses, derived from human immunodeficiency virus 1 (HIV-1), can be engineered to produce viral particles that express the SARS-CoV-2 spike protein and a fluorescent or luminescent reporter protein.[34,35,38] Crawford and colleagues recently published their method for a viral neutralisation assay based on these spike-pseudotyped lentiviral particles, where the neutralising activity of convalescent plasma was measured using luciferase in a 96-well format.[34] Schmidt *et al.* have also developed alternative HIV-1-based pseudotyped virions and a replication competent vesicular stomatitis virus (VSV)/SARS-CoV-2 chimaeric virus.[38] This VSV/SARS-CoV-2 chimaera contained the SARS-CoV-2 spike protein and green fluorescent protein (GFP) genes, and allowed neutralising activity to be measured by quantification of GFP which correlated with the reference standard SARS-CoV-2 virus neutralisation assay.[38] Such methods offer an accessible, low-risk, sensitive and specific alternative to live-virus neutralisation assays.

CONCLUSION

Virus isolation remains an important skill to be maintained for use in diagnostic laboratories, and is important for characterisation of emerging and existing viruses such as SARS-CoV-2. The pandemic virus SARS-CoV-2 grows to high titres in Vero/Vero E6 monkey kidney cells, and several human cell lines. While Vero and Vero E6 cells are easy to grow and infect with the virus, human cell lines may allow for study of human susceptibility to SARS-CoV-2 infection. Cell lines can also be engineered to overexpress the ACE2 receptor, and new methods such as spike-pseudotyped lentiviral particles for use in novel neutralising antibody assays are being developed that promise to be rapid and high-throughput, and complementary to standard ELISA. Nucleic acid testing is one aspect of the whole COVID-19 infectious picture, but not the only aspect. SARS-CoV-2 virus culture has the capacity to add

important information to the overall picture, and can inform algorithms regarding infectious capacity of an individual, and likelihood of spread.

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