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Short communication

Concentration of infectious SARS-CoV-2 by polyethylene glycol precipitation

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ABSTRACT

The development of medical countermeasures against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) requires robust viral assays. Here we have adapted a protocol for polyethylene glycol (PEG)-mediated precipitation of SARS-CoV-2 stocks without the need for ultracentrifugation. Virus precipitation resulted in a ~1.5 $\log_{10}$ increase in SARS-CoV-2 titres of virus prepared in VeroE6 cells and enabled the infection of several immortalized human cell lines (Caco-2 and Calu-3) at a high multiplicity of infection not practically achievable without virus concentration. This protocol underscores the utility of PEG-mediated precipitation for SARS-CoV-2 and provides a resource for a range of coronavirus research areas.

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2, family *Coronaviridae*) is the causative agent of the current coronavirus disease (COVID-19) pandemic. Since emerging in Wuhan, Hubei province, China in December 2019 (Zhu et al., 2020), SARS-CoV-2 has spread across the globe, causing an estimated (as of 3 August 2020) 17.7 million infections and over 680,000 deaths (World Health Organization, 2020a). There are currently no FDA-approved medicines or therapies available to treat COVID-19, with most mitigation strategies focused on physical distancing and other public health measures (Wilder-Smith and Freedman, 2020). According to the World Health Organisation there are over 120 vaccine candidates currently in various stages of development (World Health Organization, 2020b).

The development of several countermeasures against COVID-19, including antiviral compound screening, vaccine virus production and host-virus interactive studies, are reliant on robust viral assays that can be rapidly implemented in diverse laboratory settings. SARS-CoV-2 is often propagated in African green monkey kidney epithelial (VeroE6) cells for several reasons, including visible cytopathic effect and the VeroE6 deficiency in type I interferon secretion (Emeny and Morgan, 1979), which allows the virus to grow to higher titres. The tropism and replication kinetics of SARS-CoV-2 has been investigated in a range of primary and immortalized cell lines (both animal and human) (Chu et al., 2020), and while SARS-CoV-2 replication is observed in several immortalized human cell lines (notably Caco-2 and Calu-3), virus growth is relatively poor compared to VeroE6 cells. This relatively low level of virus growth in human cells may hinder efforts to develop antivirals against CoV-2, particularly when high multiplicity of infection (MOI) is required.

High molecular weight polyethylene glycol-6000 (PEG) has been used for the concentrating of virus samples for decades. Initially developed for arbovirus (Horzinek, 1969) and bacteriophage purification (Yamamoto et al., 1970), PEG precipitation has subsequently been adapted to many RNA viruses including influenza virus (*Orthomyxoviridae*) (Polson et al., 1972), respiratory syncytial virus (*Paramyxoviridae*) (Gias et al., 2008) and **HCoV-229E** coronavirus (*Coronaviridae*) (Warnes et al., 2015). While there are several alternative approaches for virus concentration, including ultracentrifugation, PEG precipitation is appealing due to its simplicity and the precipitation of virus in low-temperature, high-salt environment that stabilizes viral particles and produces a carrier medium that is isotonic to cells. Given that infectious SARS-CoV-2 is handled at high containment, it is important that research protocols are compatible with constraints of working in a physical containment (PC)-3 or PC-4 laboratory.

This method describes SARS-CoV-2 propagated in VeroE6 cells (ATCC CRL-1586) that were maintained as described previously (Stewart et al., 2020). All virology work was conducted at the CSIRO Australian Centre for Disease Preparedness at PC-4. The isolate of SARS-CoV-2 (BetaCoV/Australia/VIC01/2020) was received from the Victorian Infectious Disease Reference Laboratory (VIDRL, Melbourne, Australia) and passaged in VeroE6 cells for isolation, followed by passaging in VeroE6 cells for stock generation. All virus stocks were aliquoted and stored at −80 °C for inoculations. Other reagents required

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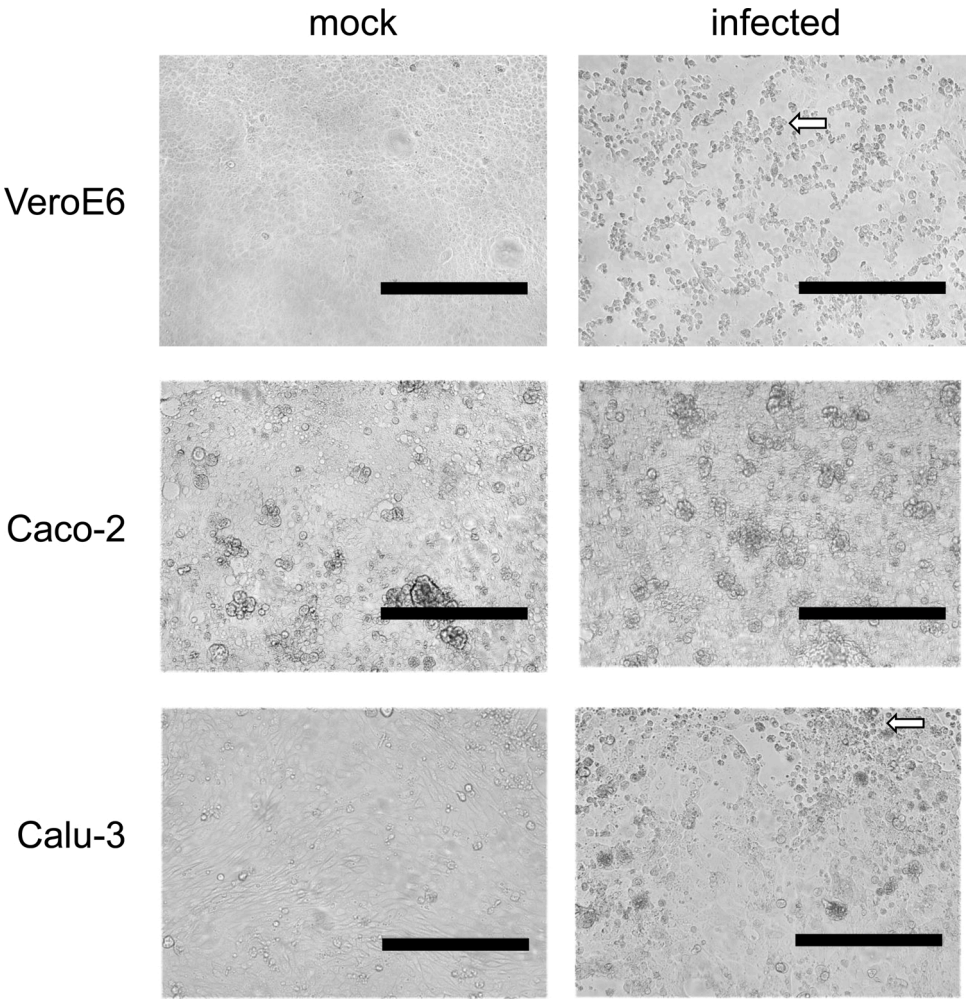

Fig. 1. Cytopathic effects of SARS-COV-2 infection in VeroE6 and Calu-3 cells, characterized by cell rounding and a granular appearance (indicated by a white arrow). No CPE was observed in Caco-2 cells. Mock-infected and infected monolayers were observed with bright- field microscopy at 48 h post-infection. Scale bar 400 μM.

for this protocol are:

- 50 mL sterile conical tubes rated to 3600 x g
- Sterile filtered 2.5 M MgSO4
- Sterile filtered 2 M Tris-HCl (pH 7.5)
- Sterile filtered NT buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.5) (stored at 4 °C)
- Sterile filtered 50 % PEG-6000 in NT buffer (store at 4 °C)
- Sterile filtered 0.5 M EDTA

SARS-CoV-2 stocks were propagated in VeroE6 cells in T150 cm^2 flasks. After 72 h of virus propagation, both cells and tissue culture supernatant (TCSN) were frozen at −80 °C overnight to lyse cells.

Upon thawing, infected cell suspensions were clarified by centrifugation 3250 × *g* for 20 min at 4 °C. 50 mL viral supernatants were adjusted to 100 mM $MgSO_4$ by the addition of 2 mL of 2.5 M $MgSO_4$ stock solution. The concentration of Tris–HCl (pH 7.5) was adjusted to 50 mM by the addition of 1.25 mL 2 M Tris-HCl (pH 7.5) stock solution. 5.5 mL of 50 % (w/v) PEG-6000 in NT was added to the clarified culture fluid to a final concentration of 10 % (v/v). Virus stocks were precipitated by incubation for 90 min at 4 °C with gentle agitation using a magnetic stirrer). Supernatants were then centrifuged at 3250 × *g* for 20 min at 4 °C and the residual supernatant removed. The pellet of precipitated virus was re-suspended in 1 mL of cold NT buffer. 2 μL of 0.5 M EDTA was added to adjust concentration to 1 mM EDTA for virus disaggregation.

The infectious titres of SARS-CoV-2 stocks, both before and after PEG-precipitation, was determined by $TCID_{50}$ assays performed as described previously (Stewart et al., 2013). Samples were titrated in quadruplicate in 96-well plates, co-cultured with VeroE6, Caco-2 or Calu-3 cells for four days. VeroE6 cells and Calu-3 cells displayed cytopathic effect, consistent with previous findings (Fig. 1). As cytopathic effect was not observed in Caco-2 cells, virus titres were measured by immunofluorescence (Fig. 2). For this, cells were fixed for 30 min in 4 % paraformaldehyde (PFA) and stained with a polyclonal antibody targeting the SARS-CoV-2 Nucleocapsid (N) protein (Jomar Sciences, catalog number: 40588-T62, used at 1/2,000) for 1 h. Cells were subsequently stained with 1/1000 dilution of an anti-rabbit AF488 antibody (Invitrogen catalog number A110008). Nuclei were counter-stained with DAPI. Cells were imaged using the CellInsight quantitative fluorescence microscope (Thermo Fisher Scientific) at a magnification of 10 x, 49 fields/well, capturing the entire well. The infectious titre for all three cell types was calculated by the method of Reed and Muench (Reed and Muench, 1938). Unconcentrated virus titres were highest in VeroE6 cells, followed by Calu-3, then Caco-2 cells (Fig. 3). Greater SARS-CoV-2 infection in Calu-3 cells over Caco-2 is consistent with previous comparisons of replication kinetics analysed by qRT-PCR (Chu et al., 2020), and may be related to the higher levels of the SARS-CoV-2 entry receptor, ACE2, in Calu-3 cells (data not shown). For each of the three cell lines, PEG precipitation resulted in a ~1.5 log_{10} increase in infectious

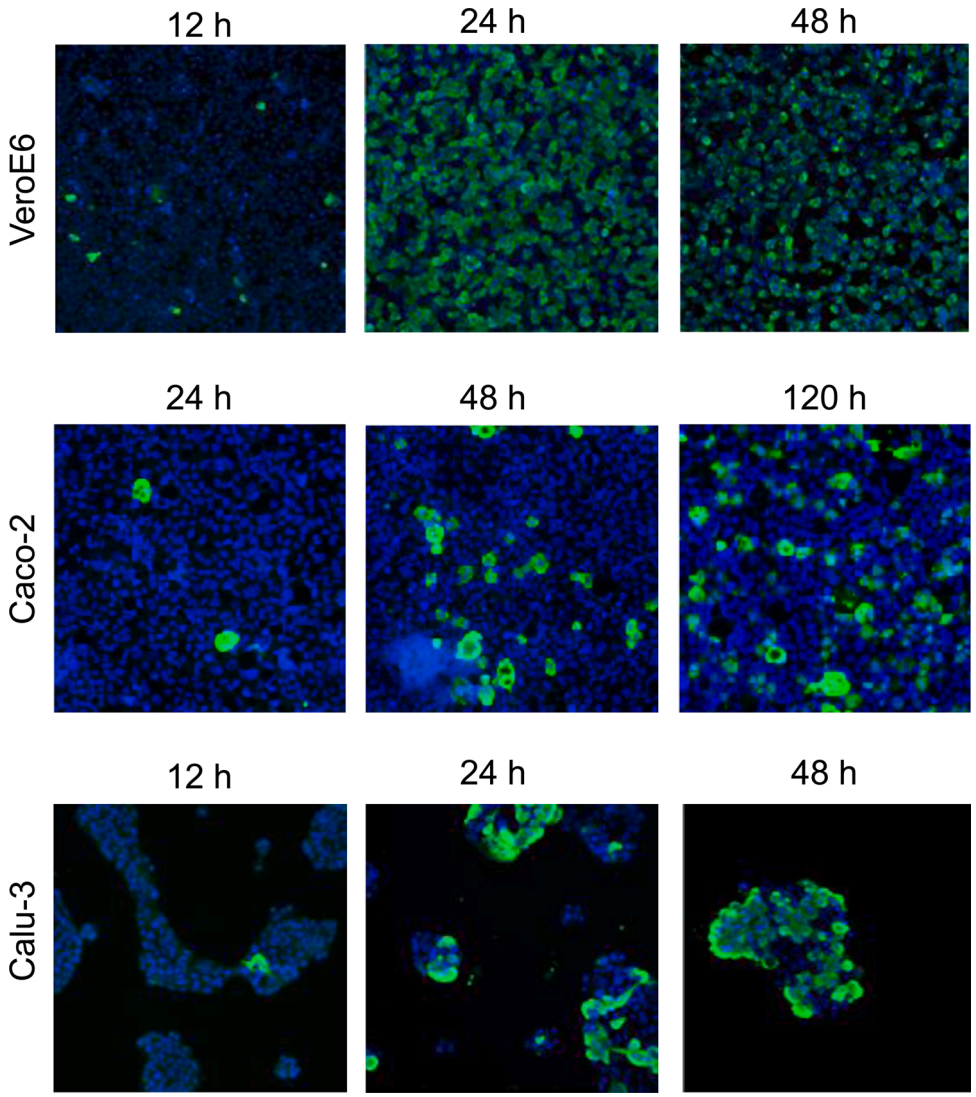

Fig. 2. SARS-CoV-2 infection of VeroE6, Caco-2 and Calu-3 cells imaged using automated fluorescence microscopy. Nuclei are shown in blue (DAPI) and SARS-CoV-2 nucleoprotein (N) in green (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

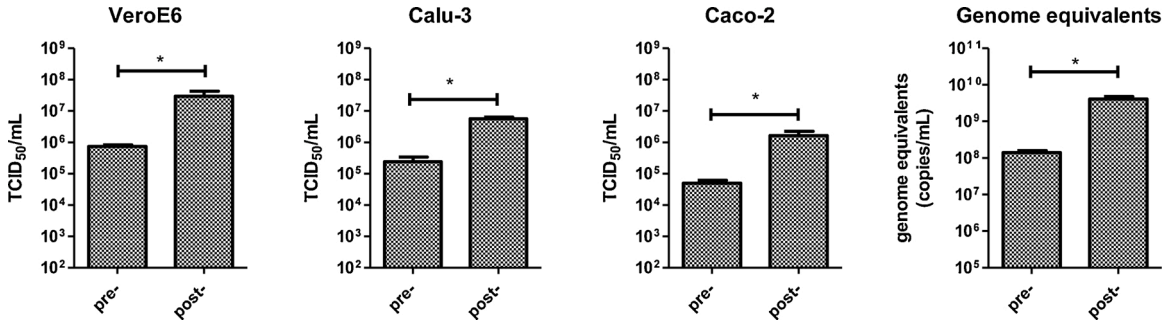

Fig. 3. TCID50 virus titres of supernatants derived from VeroE6, Calu-3 and Caco-2 cells infected with SARS-CoV-2 stocks pre- and post-PEG precipitation. Genome equivalent (viral copies/mL) as measured by qRT-PCR analysis of SARS-CoV-2 stocks pre- and post-PEG precipitation. *$p \leq 0.05$ using Student *t*-test.

virus titre. Average infectious virus titres were 7.3×10^5, 2.4×10^5 and 4.9×10^4 $TCID_{50}$/mL in VeroE6, Calu-3 and Caco-2 cells, respectively, pre-PEG precipitation. Post-precipitation, average infectious virus titres were 2.9×10^7, 5.6×10^6 and 1.6×10^6 $TCID_{50}$/mL, respectively. These results were validated using a qRT-PCR assay (Corman et al., 2020) which showed that PEG precipitation resulted in an increase of genome equivalent copies from 1.4×10^8 to 4×10^9 copies/mL.

In summary we have demonstrated that PEG-precipitation is an effective method for the concentration of infectious SARS-CoV-2 derived from tissue culture cell supernatant. The protocol described here can be carried out in a timely manner in a high-containment laboratory and does not require ultracentrifugation. Furthermore, the yield of virus is sufficient for virological assays involving high MOI in several human cell lines. As the COVID-19 pandemic continues, so does the urgent need for

robust assays for medical countermeasure development. This simple protocol can readily be scaled up and deployed to aid antiviral screening programmes.

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CRediT authorship contribution statement

Marina R. Alexander: Conceptualization, Formal analysis, Writing - review & editing. **Christina L. Rootes:** Data curation, Formal analysis, Writing - review & editing. **Petrus Jansen van Vuren:** Data curation, Formal analysis, Writing - review & editing. **Cameron R. Stewart:** Data curation, Formal analysis, Project administration, Supervision, Writing-original draft.

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