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Short communication

Evaluation of a simple ultrafiltration method for concentration of infective canine parvovirus and feline coronavirus from cell culture supernatants

Md Anik Ashfaq Khan^a, Klaus Schoene^b, John Cashman^c, Ahmed Abd El Wahed^{a,*}, Uwe Truyen^a

^a Institute of Animal Hygiene and Veterinary Public Health, University of Leipzig, 04103 Leipzig, Germany

^b Sartorius Lab Instruments, 37079 Göttingen, Germany

^c Sartorius UK Ltd., Epsom KT19 9QQ, United Kingdom



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ABSTRACT

Enrichment of viral infectious titers following its propagation by cell culture is desirable for various experimental studies. The performance of an ultrafiltration (UF) process to concentrate infectious titers of non-enveloped Canine parvovirus 2 (CPV-2) and enveloped Feline coronavirus (FCoV) obtained from cell culture supernatants was evaluated in this study, and compared with ultracentrifugation (UC) process. A mean gain of $> 1.0 \log_{10}$ TCID₅₀/mL was obtained for CPV-2 with UF, which was comparable with the gain obtained by UC. On the other hand, the gain was lower ($0.7\text{--}1.0 \log_{10}$ TCID₅₀/mL) for FCoV with UF in contrast to UC ($> 2.0 \log_{10}$ TCID₅₀/mL). However, the lower retentate volume following UC (~120 fold) compared to that following UF (~10 fold) for either of the viruses suggests a trend of increased infectious titer retention in UF concentrates relative to UC concentrates. The simplistic UF process evaluated here thus has the potential for use in applications requiring increased infectious titers of CPV-2 and FCoV.

Coronaviridae and *Parvoviridae* families comprise some of the important pathogenic viruses of human and animal species. Coronaviruses (CoVs) are enveloped, positive-sense (+) single-stranded (ss) RNA viruses that can infect a wide variety of species, including human beings and companion animals. Feline coronavirus (FCoV) is a large, spherical virus particle and belongs to the genus alphacoronavirus, which groups virions around 120–160 nm in diameter. It is, however, of phylogenetically distinct origin from that of the recent pandemic-causing severe acute respiratory syndrome coronavirus 2 (SARS CoV-2), which belongs to the betacoronavirus genus. Nevertheless, FCoV variants are known to cause symptomatic infections in cats that can result in mild to fatal disease (Drechsler et al., 2011). Furthermore, experimental FCoV infection can be used as a disease model to study coronavirus-associated immunopathogenesis (Groot-Mijnes et al., 2005). Similarly, parvovirus - a non-enveloped virus in the *Parvoviridae* family - is also known to cause infections in humans and in animals. Among different parvovirus strains, canine parvovirus 2 (CPV-2) - a small ssDNA virus with icosahedral symmetry (20–26 nm in diameter) - is assumed to have emerged following an exemplary cross-species transmission event (Lee et al., 2019) that induced a worldwide pandemic in non-immune dog

populations (Parrish and Kawaoka, 2005). Infection with CPV-2 can lead to hemorrhagic enteritis, severe diarrhea, vomiting, and leukopenia.

Experimental studies involving viruses often require viral stocks with high concentrations. While cell culture-based viral propagation systems are well developed for many viruses including CPV and FCoV, generating sufficient concentration of infective virus particles is not always possible by cellular propagation alone (Ichim and Wells, 2011; Richard and Aubry, 2018). Common virus concentration methods are based on high speed centrifugation, filtration, chemical precipitation, adsorption and affinity mechanisms; however, efficiency of the selected method is likely dependent on chemical and structural properties of the virus, condition of the sample/medium in which viral particles are retained, and the experimental purpose (Ahmed et al., 2015; Boffill-Mas and Rusiñol, 2020; Ye et al., 2016). Many of these procedures are time consuming and may require prior treatment of the sample, which can result in unwanted loss in infectious virus titer (Deboosere et al., 2011; Saha et al., 1994). Ultrafiltration (UF) is a technique commonly used to concentrate macromolecules in liquid samples. It can be independent of sample treatment steps and thus likely retains the chemical and structural properties of virus (Wyn-Jones and Sellwood, 2001). In this report,

* Correspondence to: Institute of Animal Hygiene and Veterinary Public Health, An den Tierkliniken 43, 04103 Leipzig, Germany.
E-mail address: ahmed.abd_el_wahed@uni-leipzig.de (A. Abd El Wahed).

a convenient UF method using Vivaflow 50 (Sartorius, Stonehouse, UK) equipped with a polyethersulfone (PES) membrane having molecular weight cut-off (MWCO) of 100 kDa was evaluated against ultracentrifugation (UC) for the concentration of CPV-2 and FCoV viruses from cell culture supernatants. Polymers like PES typically form an interconnected structure of nanoscale voids with sufficient pore size to retain even very small virus particles, while allowing the sample solvent (in this case, cell culture media) to permeate (Leisi et al., 2021). The efficiency of each concentration method evaluated in this study was determined from virus titration, calculated by the Spearman and Karber method (Hamilton et al., 1977) and expressed as the 50% tissue culture infectious dose (TCID₅₀) per milliliter.

CPV-2 (strain-d, GenBank: M12998.1) and FCoV (field isolate) were initially propagated on Crandell-Rees Feline Kidney (CRFK) cells in Dulbecco's medium (DMEM: 3.7 g/L NaHCO₃, 4.5 g/L D-glucose, 0.5 g/L L-glutamine) supplemented with 5% fetal calf serum (FCS, PAA Laboratories, Pasching, Austria), 1% Penicillin-Streptomycin and 1% non-essential amino acids. The propagation was conducted at 37 °C in 5% CO₂ for 6–7 days for CPV-2 and 3 days for FCoV. Virus-containing

supernatants were clarified by two successive centrifugations under 3200g at 4 °C for 10 min, with pellets discarded after each round. An initial volume of 500 mL from each of the CPV-2 and FCoV supernatant stocks were processed according to the manufacturer protocol using Vivaflow 50 (VF50; single use) or Vivaflow 50R (VF50R; multi use) tangential flow filtration (TFF) cassettes, connected to a peristaltic pump (~2 bar inlet pressure) at room temperature, and concentrated approximately 10-fold to obtain 50 mL of retentate. For comparison with UC, aliquots of 216 mL of CPV-2 and 144 mL of FCoV stock supernatants were added to centrifuge tubes (36 mL per tube). The stocks were then centrifuged in a Beckman Optima XPN-100 Ultracentrifuge with SW-32 Ti rotor at 141,400 g for 2 h at 4 °C and 100,000 g for 1 h at 4 °C for CPV-2 (Liu et al., 2020) and FCoV (Ahmed et al., 2020) (Fig. 1A), respectively. The resulting pellets in each tube were re-suspended in 300 µL of the modified Dulbecco's medium after separating the supernatants. For maximal gain, re-suspended concentrates following UC were pooled to obtain a final volume of 1.8 mL of CPV-2 and 1.2 mL of FCoV. Infectious virus titers in the retained (concentrate) and cleared (filtrate/supernatant) medium following UF and UC were determined using

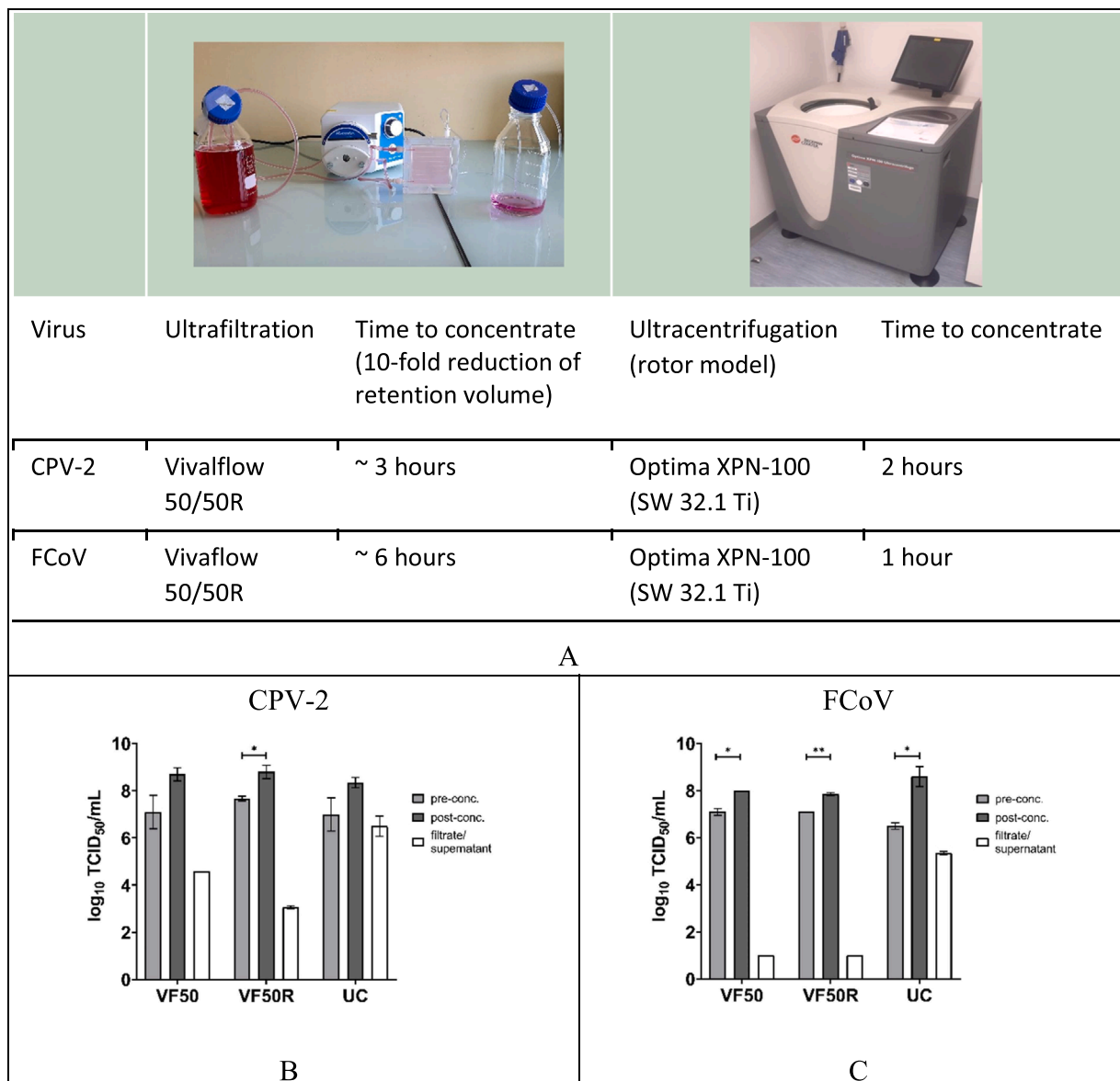


Fig. 1. Comparative methodological features (A) and performance evaluation (B-C) of UF (VF50 and VF50R) and UC methods. Infectious virus titers of CPV-2 (B) and FCoV (C) were measured from pre-concentration stock, post-concentration retentate (re-suspended pellet in case of UC) and filtrate (supernatant in case of UC). * p < 0.05; ** p < 0.01.

TCID₅₀ measurements, either by direct microscopy (FCoV) or by indirect immunofluorescence assessment (CPV-2) of the virus-specific cytopathic effects (CPE), and compared it against the CPE of stock (unprocessed) supernatants. For this, each sample was titrated by inoculating CRFK cell culture with the virus (10-fold dilution series) in 96-well plates, followed by 5 days of incubation at 37 °C in a 5% CO₂ atmosphere, as described above. For CPV-2 immunofluorescence assessment, cells were fixed on the plates with cold acetone/methanol diluted at 1:1, incubated for 15–20 min and then blocked with 3% FCS. A mix of CPV-specific monoclonal antibodies (Parrish et al., 1987) was applied for binding to CPV antigens, and then 50 µL of FITC-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories Inc., PA, USA) at a dilution of 1:100 was added to each well for detection under fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Titration assays for both CPV-2 and FCoV were performed in duplicate for each concentrated sample, with infectious virus titers from stock supernatants and filtrates also measured in parallel. To compare between and among virus titers, parametric or non-parametric tests were performed based on distribution of the data, and a two-tailed p-value < 0.05 was considered for a difference to be statistically significant. Statistical analysis and graphical representations were performed using GraphPad Prism v.8 (GraphPad Software LLC, San Diego, CA, USA).

CPV-2 infectious titers in the stock supernatants ranged from 6.60 to 7.75 log₁₀ TCID₅₀/mL (mean±SD: 7.39 ± 0.53 log₁₀ TCID₅₀/mL), with no significant difference between the stocks used for concentration by each of the chosen procedures. Post-concentration CPV-2 titer gained through both methods were in the range of 8.2–9.0 log₁₀ TCID₅₀/mL. Although the mean gain was > 1.0 log₁₀ TCID₅₀/mL by either method, there was no significant difference in the titer among the concentrates obtained through UF with VF50 (8.7 ± 0.28 log₁₀ TCID₅₀/mL) or VF50R (8.8 ± 0.28 log₁₀ TCID₅₀/mL) and UC (8.4 ± 0.21 log₁₀ TCID₅₀/mL), despite the latter yielding a final sample that is theoretically more concentrated based on final volume. In keeping with these results, mean virus titer in the UF filtrates (3.57 ± 0.9 log₁₀ TCID₅₀/mL) was lower than that of the UC supernatants (6.5 ± 0.42 log₁₀ TCID₅₀/mL) (Fig. 1B). Similarly, for FCoV, both concentration methods yielded virus titers ranging from 6.40 to 7.20 log₁₀ TCID₅₀/mL, increasing to 7.8–8.9 log₁₀ TCID₅₀/mL. However, the titer gains in concentrates from any UF cassettes (VF50 and VF50R) were lower (0.7–1.0 log₁₀ TCID₅₀/mL) than those attained by UC (> 2.0 log₁₀ TCID₅₀/mL). Despite this, the FCoV virus titers in UF filtrates were negligible (~1.0 log₁₀ TCID₅₀/mL) compared to the much higher titers (p < 0.0001) observed in UC supernatants (5.35 ± 0.1 log₁₀ TCID₅₀/mL) (Fig. 1C).

The Vivaflow ultrafiltration procedure was evaluated for its ability to feasibly concentrate infective virus particles from cell culture supernatants. The cyclic TFF process utilized by these cassettes enables continuous retention of viral particles from the sample as it is recirculated through the cassette, while the cell culture media - devoid of viral particles - permeates the UF membrane. In this study, the stock supernatants were reduced 10-fold by UF in terms of volume, thereby concentrating the viral load in theory by the same margin. On the other hand, UC concentrates were prepared by re-suspending virus-containing pellets in smaller volumes of media, resulting in a final retention volume about 120-fold lower than the initial stocks. Despite the differences in final volume (retentate or re-suspended pellet), we found comparable infectious titers in the retentates of both viruses regardless of the concentration method, which indicates increased retention of viable and infective particles in UF concentrates compared to concentrates prepared by UC. The lower recovery of infective viruses after UC can be attributed to virus inactivation by the high centrifugal forces (Lawrence and Steward, 2010). Furthermore, survival and partitioning behavior of enveloped and non-enveloped viruses in aqueous environments may vary depending on the functional groups present on their outer surface (Gundy et al., 2008; Shigematsu et al., 2014). Since CPV-2 and FCoV differ in their virion sizes (~20 nm vs. ~150 nm) as well as by the

presence of a viral envelope (non-enveloped vs enveloped), these features can lead to differences between the two viruses in terms of infectious titer gains in the concentrates and supernatants following UC. In accordance with this, when UF was used FCoV seemed to be cleared from the filtrate in terms of virus titer more prominently (p < 0.05) although slowly compared to CPV-2 (Fig. 1B and C). Together with lower gains of FCoV viral concentrate and filtrate than that of CPV-2, this suggests increased adsorption and trapping of FCoV, leading to blockage of the membrane pores, which can be followed by poor recovery (Goswami and Pugazhenth, 2020). On the other hand, and in comparison to FCoV, retention of CPV-2 by the membrane seemed poor, which resulted in a substantial virus titer loss in the filtrate. It is thus advisable to consider the virion size and ensure that an optimal MWCO is selected when choosing UF devices, to achieve optimum retention, concentration and recovery of a target virus for subsequent analyses. Nevertheless, considering the trend of substantial gain of infectious CPV-2 and FCoV virus titers, the chemical-free UF method evaluated here can be recommended as a feasible and rapid method of concentration of these viruses from cell culture supernatants for use in experimental studies, and possibly for concentrating other viruses of interest required to retain optimum functional status in the concentrate. In gene therapy research for example, UF has been used to concentrate viral vectors such as adeno-associated virus and lentivirus from cell-free matrices, facilitating the purification strategy or final drug formulation (Chakrabarti et al., 2020; Soldi et al., 2020). In the field of environmental research, UF can also be used to concentrate aquatic viruses to increase the sensitivity of detection assays, such as for the detection of porcine respiratory coronavirus to study its stability in water (De Rijcke et al., 2021). Further research can shed more light on the extent of applicability of the UF method evaluated here for obtaining infective/-functional concentrate of viruses from diverse liquid matrices.

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CRediT authorship contribution statement

Md Anik Ashfaq Khan: Investigation, Formal analysis, Writing – original draft. **Klaus Schoene:** Resources, Writing – review & editing. **John Cashman:** Visualization, Writing – review & editing. **Ahmed Abd El Wahed:** Conceptualization, Methodology, Visualization, Supervision. **Uwe Truyen:** Resources, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors except Klaus Schoene and John Cashman are in the public research sector. Mentioned authors are employed by Sartorius. However, this does not alter the authors' adherence to the scientific policies on sharing study results, data and materials.

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