

# Efficient purification of cell culture-derived classical swine fever virus by ultrafiltration and size-exclusion chromatography

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**Abstract** Large-scale production of cell culture-based classical swine fever virus (CSFV) vaccine is hampered by the adverse reactions caused by contaminants from host cell and culture medium. Hence, we have developed an efficient method for purifying CSFV from cell-culture medium. Pure viral particles were obtained with two steps of tangential-flow filtration (TFF) and size-exclusion chromatography (SEC), and were compared with particles from ultracentrifugation by transmission electron microscopy (TEM), infectivity and recovery test, and real time fluorescent quantitative PCR (FQ-PCR). TFF concentrated the virus particles effectively with a retention rate of 98.5%, and 86.2% of viral particles were obtained from the ultrafiltration retentate through a Sepharose 4 F F column on a biological liquid chromatography system. CSFV purified by TFF-SEC or ultracentrifugation were both biologically active from  $1.0 \times 10^{-4.25}$  TCID<sub>50</sub>·mL<sup>-1</sup> to  $3.0 \times 10^{-6.25}$  TCID<sub>50</sub>·mL<sup>-1</sup>, but the combination of TFF and SEC produced more pure virus particles than by ultracentrifugation alone. In addition, pure CSFV particles with the expected diameter of 40–60 nm were roughly spherical without any visible contamination. Mice immunized with CSFV purified by TFF-SEC produced higher antibody levels compared with immunization with ultracentrifugation-purified CSFV ( $P < 0.05$ ). The purification procedures in this study are reliable technically and feasible for purification of large volumes of viruses.

**Keywords** classical swine fever virus, virus purification, tangential-flow filtration, size-exclusion chromatography

## 1 Introduction

Classical swine fever virus (CSFV) is the causative agent of classical swine fever (CSF), a highly contagious and often fatal swine disease. It is an enveloped, single-stranded RNA virus with a genome of 12.3 kb and belongs to the genus *pestivirus*, the family Flaviviridae together with bovine viral diarrhea virus (BVDV), and border disease virus<sup>[1]</sup>. Its genome consists of one large open reading frame encoding a polyprotein of 3898 amino acids which is processed into seven nonstructural proteins (N-pro, p7, NS2-3, NS4A, NS4B, NS5A and NS5B) and four structural proteins including a putative nucleocapsid protein (C) and three envelope glycoproteins (E0, E1 and E2)<sup>[2,3]</sup>.

Currently, cell culture-based CSFV vaccines are used widely in China and many other countries to prevent and control CSFV infection<sup>[4]</sup>. However, large-scale production of cell culture-based CSFV vaccine is hampered because of the adverse reactions caused by the contaminants from host cell and cell culture medium. Purification of CSFV has routinely been achieved by cesium chloride or sucrose density gradient ultracentrifugation<sup>[5,6]</sup>, yet it was reported that the harsh force of ultracentrifugation might cause damage to viruses, especially to enveloped viruses<sup>[7]</sup>, and bench scale gradient ultracentrifugation is unsuitable for larger scale processes<sup>[8]</sup>. The development of an effective method for purifying CSFV virus from cell culture during vaccine manufacturing remains a research priority. To overcome this, the use of ultrafiltration has become common in virus purification<sup>[9–14]</sup>; however, virus is only partially purified through ultrafiltration and other particulates in the culture medium are concentrated. More

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recently, the application of size-exclusion chromatography has been reported for purification of turkey coronavirus<sup>[7]</sup>, pseudotyped vesicular stomatitis virus<sup>[15]</sup>, moloney murine leukemia-derived retrovirus<sup>[16]</sup>, and equine influenza virus<sup>[13]</sup>. Size-exclusion chromatography would help remove small solutes (total inclusion; most likely fetal bovine serum, amino acids and nucleotides). Hence, in this study we combined the use of ultrafiltration with size-exclusion chromatography for the concentration and purification of CSFV and provided a promising strategy for the purification of cell culture-derived viruses.

## 2 Materials and methods

### 2.1 Cell culture, virus propagation and clarification

The virulent Shimen strain of CSFV was provided by China Institute of Veterinary Drug Control and propagated in porcine kidney cells (PK-15 cells, ATCC CCL-33). A stock solution of CSFV with  $5.5 \times 10^{-4.25}$  TCID<sub>50</sub>·mL<sup>-1</sup> was employed as the seed virus. After CSFV inoculation, PK-15 cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, UK) containing 100 U·mL<sup>-1</sup> penicillin, 100 µg·mL<sup>-1</sup> streptomycin, and 2% fetal bovine serum free from bovine viral diarrhea virus and its antibody (Hyclone, Logan, Utah, USA) at 37°C with 5% CO<sub>2</sub>. Usually, maximum virus titers were obtained 48 h post-inoculation in the supernatant fluid medium, which was then pooled. Cell debris was removed by centrifugation at  $11000 \times g$  for 10 min at 4°C with a Beckman Allegra 21R conventional high-speed centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA). Clarified supernatant was stored at -70°C and used for purification by ultracentrifugation or tangential-flow ultrafiltration (TFF).

### 2.2 Ultracentrifugation

Ultracentrifugation was carried out in a Beckman ultra-

centrifuge using a SW28 rotor spinning at  $50000 \times g$  for 3 h at 4°C. Virus-containing precipitates were dissolved by PBS buffer, and the final volume was adjusted to achieve a 25-fold final concentration. Virus concentrate was applied for virus detection by electron microscopy, FQ-PCR, infectivity and recovery test.

### 2.3 Tangential-flow filtration

Clarified samples were processed by tangential-flow ultrafiltration through a Minimate TFF capsule (Pall Corporation, East Hills, USA) with a Minimate TM membrane. This device is made of two parts: the upper sample cup with the membrane and the lower filtrate collection tube (Fig. 1). The membrane of this device was pre-washed twice with ultrapure water. Then, an ultrafiltration membrane cassette with 50 kDa nominal molecular weight limit (NMWL) was employed, and the permeate was added to the reservoir and pumped through the module. The retentate was recirculated and mixed with the medium in the reservoir as feed. A 25-fold virus concentrate was harvested finally, and samples of the feed, retentate and permeate were collected at regular intervals and analyzed by FQ-PCR.

### 2.4 Size-exclusion chromatography

Concentrated CSFV from TFF was further purified by size-exclusion chromatography (SEC) at 4°C through a Sepharose 4 Fast Flow column (Bio-Rad, Hercules, CA) equilibrated with 0.1 mol·L<sup>-1</sup> phosphate buffer (pH 7.2) using a biological liquid chromatography system (Bio-Rad, California, USA). The column was eluted with the same buffer at a flow rate of 0.3 mL·min<sup>-1</sup>, and OD<sub>280</sub> absorbance of eluents was monitored continuously. Virus eluent fractions collected after SEC purification were pooled into one sample and concentrated with YM-10 Microcon Centrifugal filter device (Millipore Corporation, Billerica, USA) at 4°C, and the final volume was adjusted to the same as the ultracentrifugation.

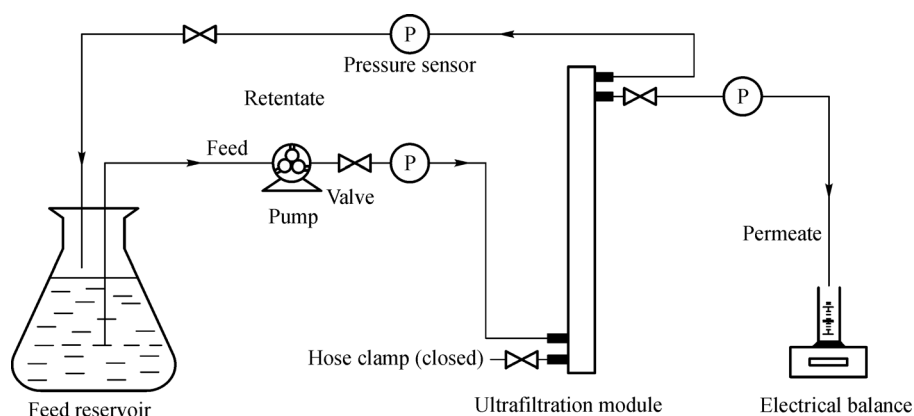


Fig. 1 Schematic representation of tangential-flow filtration

## 2.5 Virus titration

Virus infectivity before and after purification was compared by testing 50% tissue culture infectious dose (TCID<sub>50</sub>). TCID<sub>50</sub> was performed by dispensing serial 10-fold dilutions of CSFV onto PK-15 cells growing in 96-well flat bottom plates (Corning, USA). After 60 to 72 h, immunostaining of this monolayer was observed by an indirect immunofluorescence assay (IFA), and virus titer expressed as TCID<sub>50</sub>·mL<sup>-1</sup> units was calculated according to Reed and Muench method<sup>[17]</sup>.

## 2.6 Real-time fluorescent quantitative PCR

FQ-PCR was used to quantitate viral particles as described<sup>[18]</sup>. Plasmid containing the NS5A cDNA was used as an external standard for quantitation of virus particles. SYBR Green RT-PCR was performed according to the manufacturer's protocol using primers (Forward: ATTTTGCCGCATTCCAGGGC, Reverse: TCGCGTTT-GGAAGTGAAGGGA). The size of the amplicon is 149 bp. Each real-time PCR reaction (20 µL) contained 10 µL SYBR Green Real-time PCR Master Mix (TAKARA), 0.3 mmol gene-specific primers and 1 µL standardized template cDNA. Amplification was performed using an ABI 7500 thermocycler (Life Technologies, USA) with cycles as follows: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s; and final extension at 72°C for 10 min.

## 2.7 Immunoblotting

CSFV after TFF-SEC purification was applied to 12% polyacrylamide gels with sodium dodecyl sulfate (SDS). The samples were solubilized in buffer containing 62.5 mmol Tris–HCl, pH 6.8, 1% SDS, 10% glycerol, 0.001% bromophenol blue, and 1% 2-mercaptoethanol and boiled for 5 min. Polypeptide bands were revealed by staining the gel with Coomassie brilliant blue G-250. For immunoblotting, viral proteins were transferred onto nitrocellulose membrane (Millipore, Bedford, MA) at 15 V for 1 h. The nitrocellulose membrane was incubated overnight with gentle agitation in 5% (w/v) skimmed milk in PBST buffer to avoid nonspecific binding. After washing in PBST, the membrane was incubated for 1 h at room temperature with swine anti-CSFV antiserum diluted 1:200 in PBST. After washing three times HRP-conjugated goat-anti-swine IgG (Bio-Rad, Hercules, USA) was added. After incubation for 2 h at room temperature, the membrane was washed three times and covered with the 3,3-diaminobenzidine in the presence of 0.1% H<sub>2</sub>O<sub>2</sub>. The blot was allowed to develop and the reaction was stopped by washing the membrane in distilled water.

## 2.8 Electron microscopy

The presence of intact CSFV was confirmed by transmission electron microscopy (TEM). 1 µL of the purified CSFV was placed on a sheet of Parafilm. A carbon-stabilized and Formvar-coated 200-mesh copper grid was floated on the drop (film side down) and allowed to adhere to the drop for 5 min. The grid was then removed, and excess liquid was drained off by blotting the edge of the grid with a piece of clean filter paper. Then, the grid (film side down) was floated on a drop of 2% phosphotungstic acid (pH 6.5) for 1 min and air-dried for 5 min after the excess phosphotungstic acid was removed as above. Several specimens were examined under a Hitachi H-600-A2 transmission electron microscope at an accelerating voltage of 75 kV. The TEM images were recorded on Kodak electron microscope film 4489.

## 2.9 Comparison of antibody responses

Six-week-old BALB/c mice were randomly divided into three groups (A, B, C) with 5 mice per group. Group A was immunized four times at two-week intervals with  $1.0 \times 10^{-5}$  TCID<sub>50</sub>·mL<sup>-1</sup> of the inactivated purified CSFV from TFF-SEC; group B was immunized as above with inactivated ultracentrifugation-prepared CSFV; group C was injected with PBS as control. Antibody responses of each group were detected from day 0, 14, 28, 42 and 56 post-inoculation using ELISA. Briefly, microtiter plates were coated with E0-E2 fusion protein (2 µg·mL<sup>-1</sup>)<sup>[19]</sup> and incubated overnight at 4°C. A blocking solution consisting of 5% skimmed milk powder in 0.01 mol·L<sup>-1</sup> PBS containing 0.05% Tween 20 (PBS-T) was added to each well and incubated for 1 h at 37°C, then each well was washed 5 times with PBST. CSFV prepared by either TFF-SEC or ultracentrifugation produced CSFV-specific antibodies (100 µL) was added to each well and the plates were incubated for 1 h at 37°C. After washing, the secondary goat anti-mouse immunoglobulin horseradish peroxidase (HRPO)-conjugated antibody (dilution 1:1000) was added to each well and the plate was incubated for 30 min at 37°C. After washing, tetramethylene-blue (TMB, Sigma Chemicals Co.) substrate (100 µL) was added to each well and incubated for 5 min at room temperature. The absorbance values were read at 490 nm. Antibody titers from TFF-SEC or ultracentrifugation immunization groups were compared.

# 3 Results

## 3.1 Virus concentration

Virus stock was initially prepared from the cell culture supernatants and clarified by high-speed centrifugation.

Clarified supernatants were concentrated and partially purified by ultrafiltration through a 50 kDa membrane. An approximately 25-fold concentration of virus particles was obtained with a retention about 98.5% (Fig. 2a), indicating effective retention of virus particles.

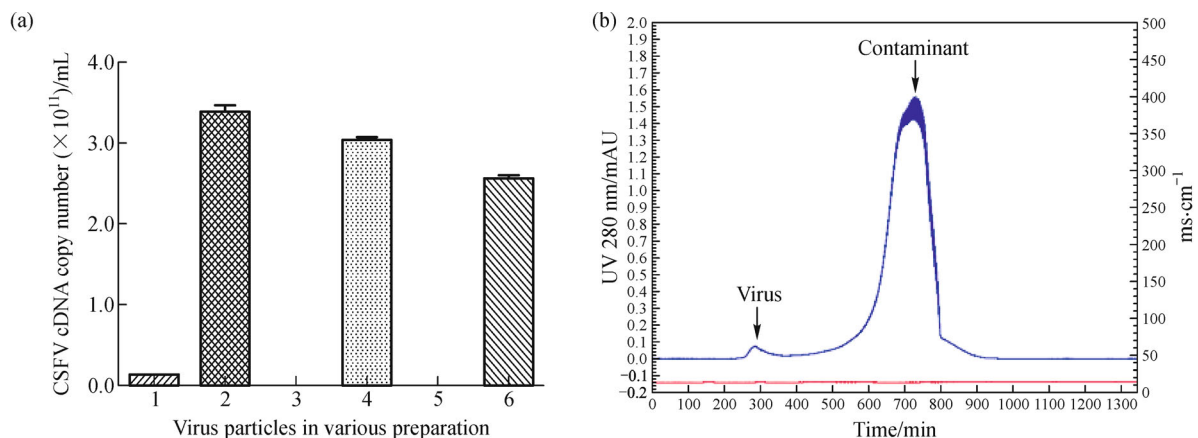
### 3.2 Size-exclusion chromatography

Two clearly separated peaks of OD280 were observed in the elution profile of size-exclusion chromatography (Fig. 2b). CSFV particles were found mainly in the first peak, and only a few viral particles appeared in the second peak. The first peak contained 86.2% of viral particles (Table 1), and the results were confirmed by SDS-PAGE and immunoblotting assay (Fig. 3). The major viral structural proteins Erns (41–44 kDa) and E1 (31–33 kDa) were detected in peak 1, the most prominent bands in the Coomassie stained gel appeared at about 70 kDa in peak 2, and previous studies suggested that this band corresponds to serum albumin precursor<sup>[20]</sup>. Virus particles were very pure and only a few faint bands of the cellular proteins could be detected on Coomassie stained SDS-PAGE in peak 1. Meanwhile, this result was confirmed by immunoblotting analyses using anti-virus antibodies

(Fig. 3b), and the viral structural proteins E1, Erns and capsid protein were detected in peak 2, while no viral proteins were detected in peak 1, which indicated the effective retention of virus particles. Meanwhile, size-exclusion chromatography served as a buffer exchange step, replacing the impurities in the cell culture medium with PBS buffer.

### 3.3 Evaluation of infectivity and recovery of virus particles

Immunofluorescence assay was used to evaluate the infectivity of virus particles purified by the TFF-SEC method or ultracentrifugation. A strong fluorescent signal was detected 48 h post-inoculation with TFF-SEC or ultracentrifugation-prepared viruses (Fig. 4), indicating that CSFV virus particles prepared by both the TFF-SEC method and ultracentrifugation were biologically active, but the titer of CSFV from TFF-SEC purification was higher ( $3.0 \times 10^{-6.25}$  TCID<sub>50</sub>·mL<sup>-1</sup>) than that obtained from ultracentrifugation ( $4.09 \times 10^{-5}$  TCID<sub>50</sub>·mL<sup>-1</sup>) (Table 1). Average infectious titer of CSFV from TFF-SEC purification was from  $1.0 \times 10^{-4.25}$  to  $1.0 \times 10^{-6.25}$ . The infectivity and recovery of the purified virus results indicated that this method was effective.



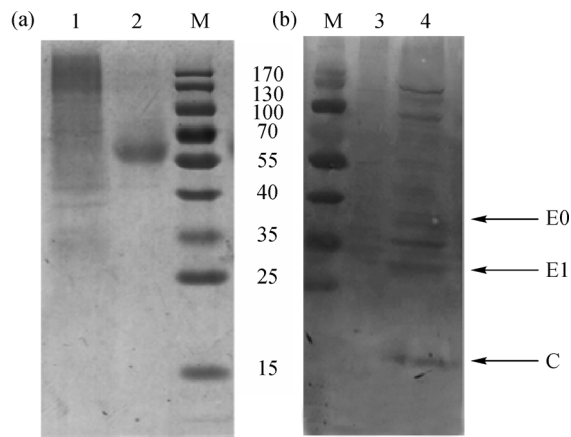
**Fig. 2** Elution profile of size-exclusion chromatography and detection of CSFV by FQ-PCR. (a) The existence of CSFV in samples at various purification stages were monitored by FQ-PCR. 1, Virus stock; 2, ultrafiltration retentate; 3, ultrafiltration penetration; 4, peak 1; 5, peak 2; 6, ultracentrifugation-prepared virus; (b) TFF-concentrated CSFV was loaded onto a Sepharose 4 Fast Flow column (100 mL bed volume). Peak 1 consisted mainly of CSFV particles and peak 2 was contaminants from host cells or cell culture media.

**Table 1** Monitoring of virus particles at various stages of purification

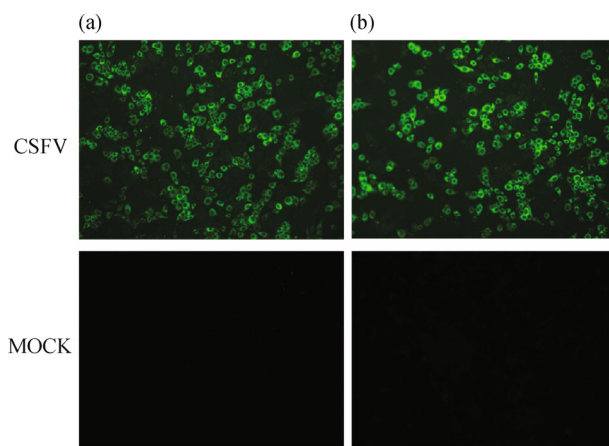
Purification stage	Total volume/mL	Particles numbers/mL	Virus recovery <sup>a</sup> /%	Virus titer /TCID <sub>50</sub> ·mL <sup>-1</sup>
Virus stock	500	$1.36 \times 10^{10}$	100	$5.5 \times 10^{-4.25}$
UF retentate	20	$3.35 \times 10^{11}$	98.5	$3.4 \times 10^{-6.35}$
UF permeate	485	N/A	N/A	N/A
Flow-through peak 1	20	$2.89 \times 10^{11}$	86.2	$3.0 \times 10^{-6.25}$
Flow-through peak 2	20	$5.07 \times 10^2$	N/A	$1.0 \times 10^{-1}$
Ultracentrifugation	20	$2.56 \times 10^{11}$	85.8	$4.09 \times 10^{-5}$

Note: <sup>a</sup> Virus recovery from each purification stage was calculated based on the total number of virus particles in the sample quantitated by FQ-PCR.

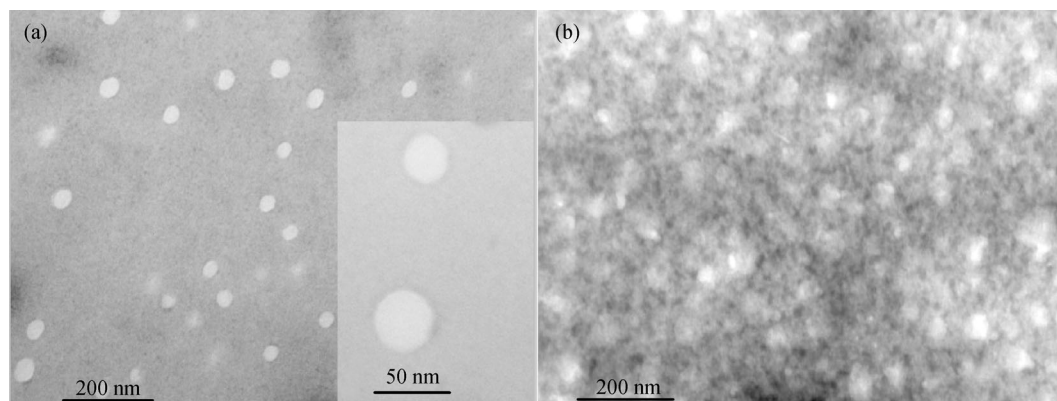




**Fig. 3** Analysis of the purified CSFV by TFF-SEC by SDS-PAGE (a) and immunoblotting (b). (a) SDS-PAGE: lane 1, peak 1 sample; lane 2, peak 2 sample; M, pre-stained protein molecular marker; (b) immunoblotting: lane 3, peak 2 sample; lane 4, peak 1 sample.



**Fig. 4** Immunofluorescence assay. (a) Monolayer of PK-15 cells infected with TFF-SEC-purified CSFV; (b) monolayer of PK-15 cells infected with ultracentrifugation-prepared CSFV. Mock-uninfected PK-15 cells were used as negative control.



**Fig. 5** Electron microscopy of the purified CSFV particles. (a) CSFV particles purified by TFF-SEC are spherical with a diameter of 50 nm; (b) CSFV prepared by ultracentrifugation show few visible intact virions and a significant amount of contaminating substances is evident as the gray background. Primary magnification of 100000 $\times$  and 300000 $\times$ .

### 3.4 Electron microscopy

The purification efficiency of the TFF-SEC method was also monitored by TEM. As shown in Fig. 5a, abundant CSFV particles with no visible contaminants were observed, and the particles were roughly spherical with the expected diameter size of 40 to 60 nm. In contrast, CSFV particles purified by ultracentrifugation gave a significant amount of protein aggregates and few visible intact CSFV particles (Fig. 5b).

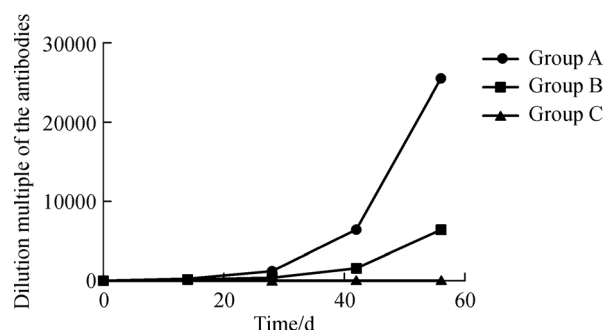
### 3.5 Comparison of antibody responses

Strip tests revealed that immunization with CSFV prepared by either TFF-SEC or ultracentrifugation produced CSFV-specific antibodies. Although, antibody levels in group A were higher than those from group B ( $P < 0.05$ ) (Fig. 6). Mice immunized with PBS in group C did not produce any antibody against CSFV.

## 4 Discussion

Virus purification is essential for producing vaccines and understanding physical, chemical, cellular and molecular biology of viruses<sup>[21]</sup>. Methods for virus purification include ultrafiltration<sup>[9,12]</sup>, size exclusion chromatography<sup>[7]</sup>, ion-exchange chromatography<sup>[11,22]</sup> and affinity chromatography<sup>[8,16,23]</sup>. In this study, a reliable and efficient method that combines tangential-flow ultrafiltration (TFF) and size-exclusion chromatography (SEC) is proposed for CSFV purification. Viral particles are separated from the contaminants on the basis of their sizes. Unlike ultracentrifugation, the methods of TFF and SEC are scalable, which make CSFV purification suitable for commercial production.

TFF-SEC purification of CSFV was compared with ultracentrifugation purification and validated. Ultrafiltration through a 50 kDa NMWL recovered 98.5% of the



**Fig. 6** Comparison of antibody responses. Antibody levels from immunization with CSFV purified from TFF-SEC (group A) and ultracentrifugation (group B) were tested with the strip test and compared. Mice immunized with PBS in group C did not produce any antibody against CSFV.

virus particles, and the yield of purified CSFV by TFF-SEC was 86.2%. This was higher than that obtained by ultracentrifugation, and the infectivity of TFF-SEC-purified CSFV was also higher than ultracentrifugation-prepared CSFV. CSFV samples prepared by both methods were biologically active and capable of infecting PK-15 cells, but TFF-SEC method removed much more contaminating proteins from the culture medium than ultracentrifugation. Electron microscopy revealed the presence of mature CSFV particles with a diameter of 40–60 nm in TFF-SEC-purified samples, and the characteristic morphology and size of purified CSFV viral particles in this study matched those described previously<sup>[24]</sup>. The antibody levels from immunization with CSFV prepared by the two methods showed significant difference ( $P < 0.05$ ), and the existence of contaminants in ultracentrifugation-prepared CSFV may reduce the efficiency of the animal's immune response.

## 5 Conclusions

We developed a reliable and efficient method for the purification of CSFV in this study. The method can be used to obtain pure virus preparations with high infectivity and no evident contamination. To the best of our knowledge, combined purification of CSFV viral particles through TFF and SEC is a novel approach. Such a purification strategy can be easily scaled for large volume production, and will help refine the purification process of enveloped viruses for vaccine production.

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**Compliance with ethics guidelines** Ruining Wang, Yubao Zhi, Junqing Guo, Qingmei Li, Li Wang, Jifei Yang, Qianye Jin, Yinbiao Wang, Yanyan Yang, Guangxu Xing, Songlin Qiao, Mengmeng Zhao, Ruiguang Deng, and Gaiping Zhang declare that they have no conflict of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of virus were followed.

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