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Effects of ultra-filtration purification of infectious bursal disease virus on immune responses and cytokine activation in specific pathogen free chickens

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Abstract

Purification is an important step in the production of viral vaccines that strongly affects product recovery and subsequent immune responses. The present study was carried out with the aim of improving the purification of infectious bursal disease virus (IBDV) by the tangential flow filtration (TFF) method. Then, the effect of the purified virus on the induction of immune responses against IBDV in specific pathogen free (SPF) chickens was investigated. The IBD07IR strain was propagated in embryonated SPF eggs. The virus was purified using a 100 kDa cassette. The quality of the recovered viruses was evaluated by titration. A total number of 60 SPF chickens were randomly divided into three groups (n = 20) and received the concentrated viral antigen, commercial live IBDV vaccine and phosphate-buffered saline at the age of 3 weeks by eye drop method. The bursa of Fabricius was examined histopathologically for possible changes. Sera were collected at 1-week intervals from day 0 until the end of 6 weeks after vaccination. The IBDV-specific antibody levels, induction of cell-mediated immunity and mRNA expression levels of cytokines were evaluated. The results showed that despite a relative raise in virus titer from 7.66 to 8.17 embryo infectious dose $(EID)_{50}$ mL^{-1} following purification, both the purified IBDV and commercial vaccine are able to induce strong immune responses against the virus. Within a context of egg-based IBDV vaccine production, a single-step TFF can be applied for the relatively purification. This platform requires a further study in the selection of multiple membranes to optimize the operating conditions and final product.

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Introduction

Infectious bursal disease (IBD) is an acute and highly contagious viral disease of young birds, which targets the lymphatic tissues and affects the bursa of Fabricius. Bursa is swollen at the beginning of the disease and may become edematous and hyperemic. Degeneration of this organ usually starts 5 days after the infection.[1] The IBD virus (IBDV) belonging to *Birnaviridae* is a double-stranded RNA and non-enveloped virus. The virus genome consists of fragment A, which encodes the viral protein (VP) 5 and the VP2-VP4-VP3 poly-protein precursor and fragment B, which encodes VP1 or viral RNA polymerase. The VP2 protein is the main antigen for the production of IBDV-specific neutralizing antibodies following vaccination and/or infection.[2,3] Live attenuated IBDV vaccines are recommended as the best way to control and prevent IBD in layer and broiler breeder farms.[4] The vaccine mimics infection to induce host immune responses and reduce immunosuppression.[5] Development of an effective vaccine builds on the knowledge of pathogen interaction with the host immune system as well as adaptive immune mechanisms. The presence of maternal antibody, nature and dose of antigen, route of administration, etc., are factors may affect the immune response to vaccination.[6] The up-regulation of T helper (Th)-1 and Th-2 immunity indicators, induction of antibody- and cell-mediated immune responses (CMI) and higher participation of innate and adaptive pathways induce protective immunity against the virus.[7,8]

Immune protection against IBDV is mainly mediated by administration of live attenuated vaccines. In live IBDV vaccine production, upstream processing comprises the propagation of the virus vaccine seed in embryonated specific pathogen free (SPF) eggs and collection of amniotic fluid and embryos. Next, debris and undesirable

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substances are removed through the primary step of the downstream process. Cell debris, host cell proteins, DNA and lipids that exist in the vaccine antigen maybe cause adverse reactions.[9] Large-scale production of the IBDV vaccine is usually hampered due to the presence of undesirable substances and contaminants derived from embryo cells. The recovery and purification of target viral antigen lead to a decrease in volume and an increase in titer which are closely connected with achieving desired final product in the entire vaccine production platform.[10]

The application of size-exclusion chromatography and ultra-filtration has been suggested for the purification and recovery of cell culture-derived viruses containing small molecules.[11,12] Tangential flow filtration (TFF) is the common technique used in ultra-filtration (UF) and provides selective separation according to the setting of the speed and filter type. The TFF has been applied extensively for the concentration and purification of propagated viruses in the downstream processing of cell-based vaccines.[13-17] The production process combined with membrane separation technology being used to produce high-quality viral stocks can greatly improve product quality by removing impurities and significantly increase production efficiency.[10] Cultivations of viruses in embryonated eggs along with the presence of large molecules in harvested allantoic fluid pose limitations for purification. The main challenge of clarification is to recover high yields of viral particles in the vaccine while effectively removing cellular debris, large aggregates and insoluble contaminants to deliver a low turbidity solution. Egg-based vaccine antigens are more difficult to purify compared to cell-based antigens due to the higher densities of allantoic fluid and impure content. Hence, the development of an effective platform for purifying IBDV during vaccine production is a priority of manufacturing.

To the best of our knowledge, the TFF method has not been used to purify the propagated IBDV in embryonated eggs. The aim of the present study was to develop a platform that can be applied to the downstream processing of egg-based vaccine purification. Toward this goal, we selected the IBD07IR vaccine strain and evaluated the impacts of the concentrated virus on the improvement of immune responses. The capacity of the purified IBDV to express adaptive immunity-related genes in the early stages following vaccination was investigated.

Materials and Methods

Infectious bursal disease virus propagation. The amount of 0.10 mL of IBDV strain IBD07IR[18] was injected into the allantoic cavity of 10-day-old SPF eggs and transferred to the incubator (BINDER, Tuttlingen, Germany) with a temperature of 37.00 ˚C for 3 - 5 days. The eggs were kept overnight at 4.00 ˚C and after that, the amniotic fluid and embryos were collected.

Virus clarification and concentration. A multiple-step operation is required for the removal of undesirable materials, especially rudimentary embryo tissue compounds. At primary clarification, low-speed centrifugation at 4,000 *g* was applied for 20 min. The larger particles were separated as pellets and the IBDV remained in the supernatant. The clarified supernatant was concentrated and purified through TFF at laboratory temperature. In this case, permeable membranes with a molecular weight cut-off of 100 kDa and permeate flux control operation were used. The inlet pressure was set to a load of 14.50377 Pa.

Virus titration. The 10^{-1} to 10^{-7} dilutions of the IBDV were prepared in phosphate-buffered saline (PBS; pH = 7.20) before and after concentration. The amount of 0.10 mL of each dilution was inoculated into five 10-day embryonated SPF eggs. After a week of incubation at 37.00 ˚C, the virus titer was calculated by the Spearman-Karber formula method and expressed as embryo infectious dose $(EID)_{50}$ mL^{-1}.

Sterility test. Standard tests to ensure the absence of microbial agents in the vaccine candidate antigen were applied according to the standard method.[19] The concentrated virus sample was diluted in sterile PBS and cultured in brain heart infusion agar, nutrient agar, nutrient broth, blood agar, tryptone soya broth and pleuropneumonia-like organisms (PPLO) agar. The media were incubated at a temperature of 30.00 - 35.00 ˚C for 1 week to check the growth of aerobic bacteria which extended to 2 weeks for PPLO agar. The thioglycolate broth was used for checking the growth of anaerobic bacteria. Sabouraud dextrose agar and broth media were used for fungi growth under incubation at 20.00 - 25.00 ˚C for 2 weeks. All of the culture media were provided from MirMedia Company (Khorramshahr, Iran).

Formulation and lyophilization. The 20.00% amount of sugar-protein stabilizer was added to the concentrated virus and the mixture was divided into type I vials after homogenization. The vials were placed in a lyophilization machine (Zirbus, Harz, Germany) and dried under freezing and vacuum conditions.

The SPF chicken immunization trial. The animal trial in this research was done to be in accordance to the Guideline for the Care and Use of Laboratory Animals in Iran [20] (Number: 98-4213).

Grouping and vaccination. Sixty day-old SPF chickens were randomly placed in three equal groups of 20, housed in an isolated and environmentally controlled room in the animal house facility for the time of 9 weeks and allowed to consume feed and water *ad libitum*. At the end of age of 3 weeks, the birds in groups A and B were vaccinated with the concentrated virus and the commercial Razi IBD vaccine (10^3 EID_{50} *per* dose; 25.00 µL), respectively, *via* eye drops based on the manufacturer's recommendation. Group C was served as a negative control. One week after

vaccination, three chickens from each group were sacrificed to observe the IBD lesions and histopathological changes in the bursa of Fabricius. The organ was aseptically removed, fixed in 10.00% neutral-buffered formalin and embedded in paraffin. Then, the blocks were sectioned in 4.00 µm thickness and stained with hematoxylin and eosin (H & E). Possible pathological changes were examined under light microscope (Zeiss, Oberkochen, Germany). Blood samples were taken from the chickens of each group before vaccination until the end of 6 weeks of the trial at 1-week intervals. The induction of immune responses was evaluated by virus neutralization (VN) and enzyme-linked immunosorbent assay (ELISA) tests for humoral immunity and T lymphocyte proliferation assay for CMI.[21] Expression profiles of immune response-associated genes in the peripheral blood mononuclear cells (PBMCs) were evaluated *via* real-time polymerase chain reaction (PCR).

Virus neutralization test. Sera of the vaccinated chickens were evaluated for the presence of specific antibodies against IBDV with an alpha VN method. Serial dilutions were prepared based on $\log_{10}$ (10^{-1} to 10^{-7}) of the virus and the fixed amount of serum was added to their equal volumes. For each dilution, five 10-day-old embryonated eggs were inoculated, kept at 37.00 ˚C for 7 days and candled every 24 hr. The endpoint was recorded as the highest dilution of the virus that did not cause any IBD specific lesions in the embryos and expressed as neutralizing index.

Enzyme-linked immunosorbent assay test. Sera of the vaccinated chickens were evaluated for the presence of IBDV specific antibodies using the IDEXX FlockChek® IBD ELISA Kit (IDEXX Laboratories, Inc., Westbrookm, USA). Briefly, 100 µL of diluted serum samples were aliquot into each well of a micro-plate and incubated for 30 min at laboratory temperature. Negative and positive controls were considered. The wells were washed three times, then 100 µL of the conjugate was added to each well and the plate was incubated again for 30 min. After washing the wells, 100 µL of 3,3′,5,5′-tetramethyl-benzidine (Sigma-Aldrich, Steinheim, Germany) substrate was poured into each well and the incubation continued for 15 min. The 100 µL of stop solution was added to each well and the optical density (OD) at 650 nm was measured using an ELISA Reader (BioTek, Santa Clara, USA). The anti-logarithm of the titer (Log_{10}) was calculated and recorded as the amount of specific antibody against IBDV for each sample.

T lymphocyte proliferation assay. The PBMCs were isolated from chicken groups on 21st post-vaccination day using lymphocyte isolation medium (Sigma-Aldrich) with specific gravity of 1.077 g mL^{-1}. The PBMCs from each group were cultured with RPMI 1640 (INOCLON, Karaj, Iran) and seeded in each well of a 96-well micro-plate at a rate of 2.00×10^5 cells mL^{-1}. To compare the proliferative response, lymphocytes were stimulated with phytohem-agglutinin as a positive control and IBDV. After 72 hr incubation at 37.00 ˚C and 5.00% CO_2, 10.00 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) solution (5.00 µg mL^{-1} of final concentration) was added to each well and then, the micro-plate was incubated at 37.00 ˚C for 4 hr. Cells were collected and incubated with 150 µL dimethyl sulfoxide (Sigma-Aldrich) to dissolve insoluble formazan crystals. The absorbance was measured at 630 nm and the mean ± standard deviation was calculated for three replicates. Stimulation index (SI) was calculated from the formula as follows:

$$SI = \frac{Mean\ OD_{\ stimulated\ lymphocytes} - Mean\ OD_{\ blank}}{Mean\ OD_{\ unstimulated\ lymphocytes}}$$

Cytokine mRNA expression level. The genomic RNA was extracted from PBMCs using RNA-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Gyeonggi-do, South Korea) on 14th and 28th post-vaccination days. The RNA was converted into complementary DNA (cDNA) by SCRIPT cDNA Synthesis Kit (Jena Bioscience, Jena, Germany). The amplification step was carried out using 10.00 µL qPCR GreenMaster (Jena Bioscience), 1.00 µL template cDNA, 0.50 µL each forward and reverse primer and RNAse-free water up to 20.00 µL. The specific primers consisted of *F: TTCTGGGACCACTGTATGCTCTT* and *R: TACCGACAAAGTGAG AATCAATCAG* corresponding amplification of 129 bp of chicken *interleukin-2 (IL-2)* gene, *F: AAGTCAAAGCCGCACAT CAAAC* and *R: CTGGATTCTCAAGTCGTTCATCG* corresponding amplification of 132 bp of chicken *interferon gamma (IFN-γ)* gene and *F: TGCCATCACAGCCACACAGAAG* and *R: ACTTT CCCCACAGCCTTAGCAG* corresponding amplification of 123 bp of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were designed. Thermal cycling profile was as follows: 50.00 ˚C for 0 min and 95.00 ˚C for 15 min followed by 40 cycles of 95.00 ˚C for 10 sec and 60.00 ˚C for 20 sec. The relative expression levels of cytokine genes were normalized using housekeeping gene *GAPDH*, and changes were expressed as n-fold higher or lower in levels relative to the control.

Statistical analysis. The statistical analyses were performed using SPSS Software (version 22.0; IBM Corp., Armonk, USA) to determine the statistical significance of the individual data point's comparison. Differences with $p \leq 0.05$ were considered significant.

Results

The IBDV titer before UF was estimated to be 7.66 EID_{50} mL^{-1} which was increased to 8.17 after applying the TFF process.

At the end of the incubation period, no colonies were observed on the culture medium plates under different conditions. Therefore, the concentrated IBDV antigen was not contaminated with bacteria, mycoplasma and fungi.

No visible signs of IBD disease were observed in the vaccinated birds during the daily inspections. At necropsy, the symptoms of the disease including bleeding of the chest and thigh muscles, increased intestinal mucous secretions, necrosis and tissue destruction of the spleen and cecal tonsils and nephritis with urates were not observed in any of the vaccinated and control birds. Following the administration of commercial vaccine and concentrated viral antigen, no signs of bursa change were observed in any of the birds in groups A and B, and this organ had normal size and appearance. In histopathology, nearly 100% of lymphoid follicles were normal in the bursa of control chickens; while, a very mild depletion of lymphoid cells was noted in vaccinated chickens (Fig. 1).

A time course of the VN IBDV-specific antibody titer in each group is shown in Figure 2A. In vaccinated chickens with concentrated IBDV antigen, an appropriate titer was established comparable to the commercial vaccine. The amount of antibodies indicated that the serum had neutralized the virus in vaccinated chickens. Raise in the VN antibody titer was detected, being increased in the 3rd week and remained high throughout the experiment. Purified IBDV showed a better antibody response up to 6 weeks after vaccination; however, overall the differences between the vaccinated groups were not statistically significant. Statistical analysis showed a significant difference ($p \leq 0.05$) in IBDV-specific neutralizing antibody titers between the vaccinated and control groups.

Serum IBDV antibody levels, as monitored by ELISA, showed similar rates of raise between the vaccinated groups (Fig. 2B). This rate was slightly higher in the group receiving the concentrated virus vaccine than the one receiving the commercial vaccine and reached 2873 and 2,729 U mL^{-1} ELISA in the 3rd weeks after vaccination, respectively. The antibody level in the negative control group was below detectable limits. Compared to the control group, such increase in IBDV antibody levels was statistically significant.

Three weeks after vaccination, CMI were measured in the vaccinated chicken groups (Fig. 3). The proliferation activity was increased significantly in vaccinated groups compared to the negative control chickens. The amount of SI in the groups A and B was estimated to be more than threshold 2 that meant the increase of T lymphocytes proliferation and CMI induction.

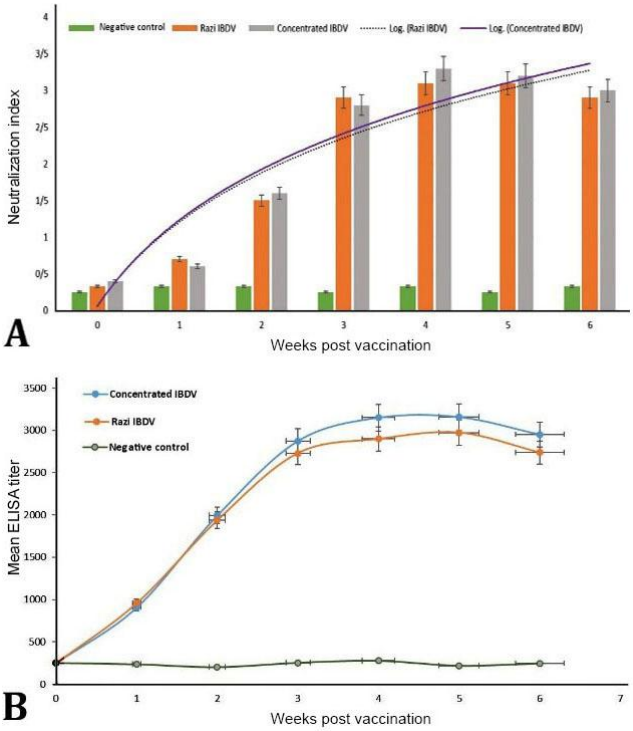

Fig. 2. Specific antibody titration against infectious bursal disease virus (IBDV) in specific pathogen free chickens being immunized with commercial vaccine and concentrated antigen. **A)** Neutralizing antibody titers in the serum obtained from chicken groups. The trend of increasing in the specific titer is shown with dotted and continuous lines; **B)** Antibody titers against IBDV measured by ELISA.

The mRNA expression levels of Th-1 and Th-2 cytokines were quantified using real-time PCR. The results showed that the expression levels of IFN-γ and IL-2 were significantly ($p < 0.05$) up-regulated in both vaccinated groups compared to the unvaccinated group at any time point (Fig. 4).

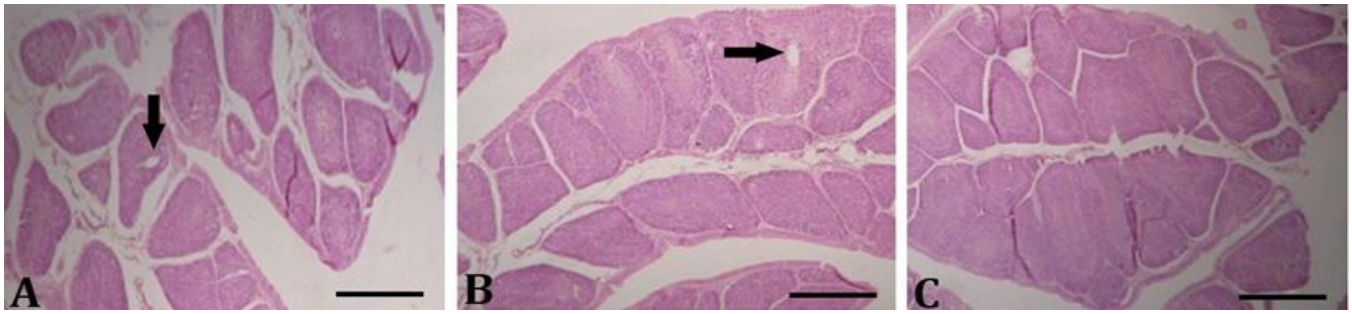

Fig. 1. Histopathological appearance of bursa of Fabricius. **A)** Mild depletion of lymphoid cells of the bursal follicles being indicated by arrow and a lot of normal lymphoid cells in the bursa of group A chickens being vaccinated by IBD07IR 1 week after vaccination; **B)** The same feature in group B being vaccinated by concentrated infectious bursal disease virus; **C)** Normal lymphoid follicle in the bursa of control chickens (H & E staining, bars = 100 µm).

The mRNA of the *GAPDH* housekeeping gene was continuously expressed in all groups. Similar to the commercial vaccine, significant increases in the levels of these cytokines indicated the ability of concentrated IBDV to induce strong immune responses against the virus.

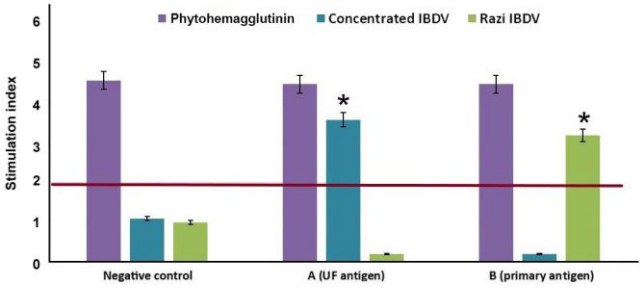

Fig. 3. Lymphocyte proliferation response to infectious bursal disease virus in specific pathogen free chickens being immunized with commercial vaccine and concentrated antigen. The stimulation index threshold limit is shown with a red line. Bars represent means ± standard errors. * indicates statistically significant differences ($p < 0.05$) between the averages found for the vaccinated groups and those found for the control group. UF: Ultra-filtration; IBDV: Infectious bursal disease virus.

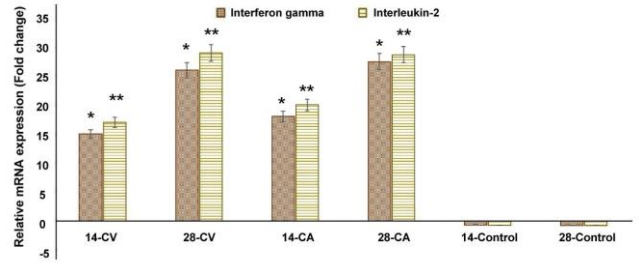

Fig 4. Relative mRNA expression levels of IFN-γ and IL-2 cytokines in SPF chickens immunized with commercial vaccine (CV) and concentrated antigen (CA) on 14th and 28th days after vaccination. Results are represented as fold change compared to levels in negative control, after normalization with GAPDH in log_2 scale. Bars show means ± standard errors. * and ** represent the significant differences ($p < 0.05$) in expression of IFN-γ and IL-2 in vaccinated groups compared to the control group.

Discussion

The IBD is a highly contagious viral infection and an important suppressor of the immune system of young chickens which is considered a threat to the world poultry industry. In order to overcome the significant economic losses of this disease, poultry vaccination has been suggested as the most important and effective tool for preventing the disease. Egg-based vaccines are the majority of IBDV vaccines currently offered on the market. Compared to other avian viral vaccines, the complex composition of IBDV vaccines makes production process technically challenging. Removing impure particles plays an important role in improving vaccine quality and optimization; accordingly, UF can help solve the challenges in IBDV vaccine production. In this study, an attempt was made to concentrate and purify the antigen containing live virus by purifying the IBDV-infected allantoic fluid and embryos using TFF for the first time. We applied a 100 kDa TFF cassette for recovery of virus particles from other components obtained in harvest step following a low-speed clarification. The success of purification with TFF depends on the type of virus and vaccine production system. The IBDV T = 13 icosahedral capsid is most probably assembled with VP2 and VP3 copies and has a diameter of about 60.00 nm.[22] Thus, the relatively small size of IBDV compared to other viruses does not cause a problem to pass through the membrane and be collected at the permeate side. Similar results have indicated that concentration and purification under influenza virus types with TFF together with a 100 kDa cassette can lead to proper performance.[23,24] A 100 kDa TFF membrane was used to concentrate the poliovirus which led to an increase in immunogenicity of the prepared inactivated vaccine as well as the vaccine shelf-life.[25] Similarly, this system was used for the measles virus grown on Vero cells and resulted in high-titer virus purification.[15] Studies on the purification of rabies virus using a 100 kDa cassette have indicated the effectiveness of TFF method in the production of human rabies vaccine.[26,27]

The TFF process operates well for substance at low to moderate impurities; however, it has challenges with high-impurity biological products. The results of research on the purification of both enveloped (influenza, measles and rabies) and non-enveloped (polio and foot and mouth) viruses propagated in cell cultures have revealed that UF can be used to produce high-titer viral stocks.[15-17,26,27] The main drawback of egg-based vaccines is the complex composition of allantoic fluid which alters vaccine production downstream processes, especially the quality and product yield. A very viscous consistency of allantoic fluid due to the presence of high amounts of protein such as ovalbumin and minerals makes it challenging part to clarify. This fluid also contains the basic tissue components of embryo such as feathers, beak and blood vessels and cells. The turbidity of a virus-containing allantoic fluid is generally 46 - 132 nephelometric turbidity unit (NTU). At the downstream process, clarification should be optimized to maximize product yield and purity to provide a recovery yield of 90.00% with a turbidity value below 10 NTU.[28,29] Despite the vast impurities of egg-based antigen suspensions, cell culture-based viruses contain levels of host cell-derived debris and secretory viral proteins. It has been shown that use of 50.00 kDa membranes also increases the recovery efficiency of influenza virus particles up to 98.50%, which has been effective in improving the levels of specific antibodies in the immunization of pigs.[30] Our preliminary data to achieve high-titer IBDV stock by TFF device showed an increase in virus titer following the concentration.

Then, the effect of the concentrated vaccine to improve immune responses was evaluated in SPF chickens. Despite a relative raise in virus titer, a positive impact on immune responses and protective capacity of the purified virus was not detected compared to the commercial vaccine. Also, the two vaccinated groups showed a similar increase in cytokine expression levels. Based on the data, it seemed that concentration of virus by TFF had a slight effect on the increased effectiveness of the vaccine and improving immune responses against IBDV in vaccinated birds. Due to the high turbidity of stock IBDV antigen and presence of host proteins, other impure particles might have been concentrated along with the virus. There is no uniform template or platform for the clarification step in the downstream process of vaccine production and its success influences by the physicochemical properties of the target virus and its replication substrate. The TFF system works by separating components from each other based on molecular size and continuous concentration, and removing excess particles. Therefore, proper selection of the device, membrane type and size, molecular weight cut-off, temperature, pH and buffer composition are critical for the high recovery of virus particles from an impure and turbid suspension. For example, to retain viral particles, the cut-off molecular weight of the filter membrane should be smaller than the particle (two times smaller than the molecular weight of the viral particle); however, large enough to filter smaller components.[31]

By comparing our data and previous studies, we could conclude that TFF operated well for substance at low to moderate impurities; however, it had challenges with high-impurity biological products. Therefore, it is suggested to maximize the benefits of TFF in the purification and concentration of IBD viral antigen by selecting the membranes with larger pore sizes where all particles of virus pass through it to remove the large proteins of the host cell, choosing the membranes with smaller pore sizes to remove the DNA and small proteins of the host cell, and finally concentrating the virus particles.

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Conflict of interest

No conflict of interest is declared.

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