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Determination of the optimal method for the concentration and purification of 146S particles for foot-and-mouth disease vaccine production

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ABSTRACT

After the severe outbreak of foot-and-mouth disease (FMD) in South Korea in 2010, the Korean government implemented a vaccination policy and set out to develop an FMD vaccine using a local FMD virus (FMDV) strain. As a part of the basic research for domestic FMD vaccine development, three methods commonly used for the concentration and purification of FMDV to produce FMD vaccine antigens were compared. Among common concentration methods, including polyethylene glycol (PEG) precipitation, ammonium sulfate precipitation, and ultrafiltration, the most effective method both for concentrating 146S particles and eliminating non-structural proteins (NSPs) was found to be PEG precipitation. Classical PEG precipitation showed the highest recovery of 146S particles (85.4%) with removing 99.8% of the other proteins, including NSPs. To the author's knowledge, this is the first study to compare the current three methods with regard to quantifying intact virus particles (146S). These findings may provide important insights for the development of new FMD vaccines using a local FMDV strain in the near future.

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cloven-hoofed animals caused by the foot-and-mouth disease virus (FMDV) belonging to the genus *Aphthovirus* and family Picornaviridae (Barteling and Vreeswijk, 1991). In November 2010, after the large-scale FMD outbreaks, the Korean government enforced immediate vaccinations to prevent additional FMD occurrences. Nevertheless, another FMD outbreak caused by the O Jincheon/SKR/2014 strain (O-JC) occurred from 2014 to 2015. In Korea, all farm animals susceptible to FMD have been vaccinated, but all the vaccines have been imported from foreign manufacturing companies. Therefore, the Korean government started planning to establish its own FMD vaccine manufacturing company in the near future.

According to Office International des Epizooties (OIE) standards, for production of FMD vaccines, it should be verified that these vaccines do not induce antibodies against non-structural proteins (NSPs) under the conditions used in the field after repeated vaccinations (Barteling, 2002). However, it is impossible to eliminate NSPs completely considering the manufacturing cost of animal vaccines, even though vaccine purity is important for differentiating infected from vaccinated animals. In addition, FMDV should be concentrated to small volumes so that it can be stored in vaccine antigen banks. There are various methods such as polyethylene glycol (PEG) precipitation (Degerli and Akpinar, 2001; Huhti et al., 2010), ammonium sulfate (AS) precipitation (Harter and Choppin, 1967), and ultrafiltration (UF) systems (Giessauf et al., 2002; Guo et al., 2009) to concentrate and purify viral antigens. Previous studies showed the concentration and purification efficiency of each method through measuring virus titer and complement fixing activity (Galloway and Schlesinger, 1937; Kaaden et al., 1971; Morrow, 1972). As previously reported, an intact FMDV particle (146S) is the most crucial factor for inducing neutralizing antibodies (Pay and Hingley, 1992). Therefore, the objective of this study was to determine the most efficient method to recover intact 146S FMDV antigens among these three well-known methods.

Baby hamster kidney (BHK-21) suspension cells (6×10^6 cells/ml) were inoculated with the FMDV O Jincheon/SKR/2014 (O-JC) strain at a multiplicity of infection (MOI) of 0.01 and then clarified virus culture supernatant was harvested at 16 hours (h) post-infection by centrifugation (4,000 rpm for 20 min). For inactivation of the clarified

Abbreviations: AS, ammonium sulfate; O-JC, FMDV O Jincheon/SKR/2014; SDG, sucrose density gradients; TN, tris-NaCl; UF, ultrafiltration

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Table 1
Comparison of total protein removal and antigen recovery of inactivated FMDV depending on the PEG concentration.

	BEI-inactivated	% PEG				
		2.5	5	7.5	10	12.5
Total protein[†] concentration (μg/Mℓ)	4237 ± 1047	1.29 ± 1.0[a,]	6.16 ± 1.2[a,b]	8.01 ± 1.6[a,b]	8.99 ± 1.5[a,b]	10.50 ± 3.1[b]
Total protein Removal (%)	0	99.98 ± 0.02[a,]	99.84 ± 0.03[a,b]	99.80 ± 0.01[a,b]	99.77 ± 0.02[a,b]	99.74 ± 0.04[b]
146S particles[†,‡] concentration (μg/Mℓ)	3.45 ± 0.41	0.08 ± 0.02[***]	2.60 ± 0.29	2.95 ± 0.35	2.07 ± 0.66[**]	0.91 ± 0.31[***]
Recovery[‡] of 146S particles (%)	100	2.30 ± 0.78[***]	75.37 ± 1.03	85.40 ± 0.30	57.2 ± 14.3[**]	25.53 ± 8.4[***]

[†] All values were processed by dividing the total amount by the respective concentration factor (BEI − inactivated FMDV culture supernatant, non − concentrated: 1; various % of PEG − concentrated samples: 50). Means with different alphabetic superscripts show significant differences at $p < 0.05$, n = 4.

[‡] Quantitation of 146S particles was performed by calculating the peak on the chart recorder of density gradient fractionator. Efficiency of 146S particle recovery compared to the BEI-inactivated supernatant group when concentrated by the respective PEG content. [**]; $p < 0.01$, [***]; $p < 0.001$, n = 3.

virus culture supernatant, 3 mM binary-ethylenimine (BEI) (Sigma-Aldrich, St. Louis, MS, USA) was added and incubated in a shaking incubator (26 °C for 24 h) (Bahnemann, 1990). Residual BEI was neutralized with 2% sodium thiosulfate (Daejung Chemicals, Siheung-si, Korea).

As a control group, 50 ml of the inactivated FMDV culture supernatant was ultra-centrifuged at 100,000 × *g* for 4 h at 4 °C with a SW41Ti rotor (Beckman Coulter, Brea, CA, U.S.A). Because the resultant pellets were resuspended again in 50 ml of TN buffer (pH 7.2), final concentration factor of the control group is 1. Otherwise, each 50 ml of the inactivated FMDV culture supernatant was concentrated by mixing with either a final concentration of 7.5% PEG 8000 or 50% AS and stirring overnight at 4 °C, as described in previous studies (Alouini and Sobsey, 1995; Barteling and Meloen, 1974; Desselberger, 1994; Iyer et al., 2000; Shirai et al., 1990). The precipitate was obtained by centrifugation (10,000 × *g* for 30 min) and resuspended in 1 ml of Tris-NaCl (TN) buffer (pH 7.2) to make the final concentration factor 50. Resuspended solution was centrifuged (10,000 × *g* for 10 min) to collect the supernatant. Then, ultracentrifugation steps were carried out at 100,000 × *g* for 4 h at 4 °C with a SW41Ti rotor (Beckman Coulter, Brea, CA, U.S.A) to standardize the amount of starting material for each concentration and purification method, and the resultant pellets were resuspended again in 1 ml of TN buffer (pH 7.2). On the other hand, 200 ml of the inactivated FMDV culture supernatant was concentrated by UF using a polyethersulfone membrane cassette with a nominal molecular weight limit of 300 kDa (EMP Millipore Corporation, Billerica, U.S.A) (Adikane et al., 1997; Barteling, 2002) until the retentate of UF reached a volume of 20 ml (final concentration factor is 10). The whole retentate of UF was ultra-centrifuged with a SW32Ti rotor (Beckman Coulter, Brea, CA, U.S.A) to standardize the amount of starting material for each concentration and purification method, and the resultant pellets were resuspended in 1 ml of TN buffer (pH 7.2). Concentration by three methods was done at the same day.

Quantification of 146S particles were done as follows. Either 1 ml of the viral suspension non-concentrated or concentrated by PEG, AS, or UF was layered onto 15–45% sucrose density gradients (SDG) and ultra-centrifuged at 100,000 × *g* for 4 h at 4 °C with a SW41Ti rotor (Beckman Coulter, Brea, CA, U.S.A). The ultra-centrifuged SDG was sucked through a hollow needle inserted to the base of the centrifuge tube and pumped through the flow cell of a spectrophotometer at 2 ml/min using a continuous density gradient fractionator connected to an UA-6 monitor (Teledyne ISCO, Lincoln, NE, U.S.A) (Doel et al., 1981). The absorbance of each fraction was consecutively recorded at 254 nm with ${E_1}_{cm}^{1\%} = 72$ at 254 nm; sensitivity 1 absorbance unit. The peak of 146S particles was monitored at a chart speed of 60 cm/h and was calculated to measure the quantity of 146S particles (μg/ml) (Doel et al., 1981). SDG ultra-centrifugation and the absorbance reading of all samples were done at the same day.

Meanwhile, the microplate bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL, U.S.A) and FMD IPC 3ABC ELISA kit (Prionics, Lelystad, Netherlands) were used to measure the total protein and NSP content, respectively in viral supernatant and concentrates using PEG precipitation, AS precipitation, and UF according to the manufacturer's instructions. The band between the 30% and 35% sucrose layers was collected and ultra-centrifuged. The pellet was dialyzed with TN buffer. One drop of the purified FMDV was placed on formvar-coated grids and stained negatively with 1% uranyl acetate. The FMDV particles were examined under transmission electron microscopy (TEM) (Hitachi 7100, Tokyo, Japan). Statistical significance was evaluated with one-way ANOVA using GraphPad Prism 5 (GraphPad Software, San Diedo, AD, U.S.A). All data are representative of three independent experiments and values are represented as the mean ± standard error of the mean (SEM).

Initially, the most effective PEG concentration for 146S antigen recovery was determined as vaccine potency increases with the increase in the 146S antigen content. The recovery of 146S particles increased gradually as the PEG concentration increased up to 7.5%. However, the 146S antigen yields showed sudden decrease with increase in PEG concentration beyond 7.5%. Thus, 7.5% was selected as the most optimum concentration of PEG for FMDV antigen recovery (Table 1). In addition, PEG precipitation showed a significant decrease in the total protein content compared to the non-concentrated BEI-inactivated group, and the amount of total proteins did not show any significant differences with the variation in PEG concentration. When PEG precipitation is used for purification and concentration of viral proteins, the optimal PEG concentration varied depending on the viruses. Focusing on the FMDV, previous studies concentrated the antigens using 6–8% PEG (Barteling, 2002; Barteling and Meloen, 1974; Hassan, 2016; Iyer et al., 2000; Shirai et al., 1990; Yang et al., 2013). The present study confirmed the PEG concentration for producing the FMD vaccine antigen by comparing 146S content; when the PEG concentration was below or above the recommended range (6–8%), it resulted in poor 146S particle recovery. Moreover, the 10% and 12.5% PEG mixtures were considered highly viscous to achieve the accurate volume. Recovery of FMDV 146S particles concentrated by 7.5% PEG was over 80% of that of the original viral supernatant, and NSP content was reduced below the detection limit, which did not induce antibody response against NSP after three vaccine administrations in goats (data not shown).

Next, PEG precipitation, AS precipitation, and UF systems were compared and analyzed to find the most efficient concentration and purification method for FMD vaccine production with O-JC (Table 2). UF systems have been used by many vaccine manufacturers to obtain highly concentrated antigens as the scale-up process is simple (Barteling and Vreeswijk, 1991; Negrete, Pai, and Shiloach, 2014) (Negrete et al., 2014). Enterovirus 71 particles (approximately 30 nm) of the Picornaviridae family similar to 146S particles of FMDV were concentrated using a 300-kDa membrane in a UF cassette (Chang et al., 2012). To select the UF membrane, the membrane pore sizes of 100 and 300 kDa were compared. As a result, the amount of 146S particles

Table 2
Methodological comparison for 146S particles and the contaminant (total proteins and NSPs) content after concentration and purification of inactivated FMDV culture supernatant.

	BEI-inactivated	7.5% PEG	50% AS	UF
146S particles[†,‡] concentration (μg/ml)	4.44 ± 0.14	3.70 ± 0.17**	2.82 ± 0.11***	1.49 ± 0.04***
Recovery[‡] of 146S particles (%)	100	83.36 ± 1.81**	63.59 ± 2.72***	33.50 ± 0.39***
Total protein[†] concentration (μg/ml)	3251 ± 234.3^{a}	15.7 ± 1.547^{b}	55.58 ± 7.594^{b}	856.8 ± 87.79^{c}
NSP[†] concentration (ng/ml)	219.1 ± 16.80^{a}	2.4 ± 0.545^{b}	4.33 ± 0.384^{b}	24.17 ± 7.427^{b}

[†] All values were arithmetically calculated by dividing the total amount by the respective concentration factor (BEI − inactivated FMDV culture supernatant, non − concentrated: 1; PEG − concentrated samples: 50; AS − concentrated samples: 50; UF − concentrated samples: 10). Means with different alphabetic superscripts show significant differences at $p < 0.05$, n = 3.

[‡] Quantitation of 146S particles was performed by calculating the peak on the chart recorder of density gradient fractionator. Efficiency of 146S particle recovery compared to the BEI-inactivated supernatant group when concentrated by other three methods. **; $p < 0.01$, ***; $p < 0.001$, n = 3.

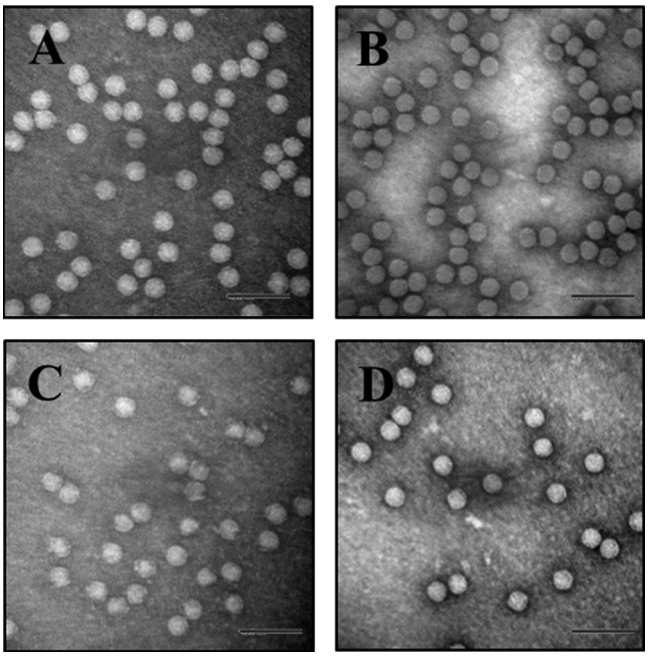

Fig. 1. Morphological intactness of viral particles verified by transmission electron microscopy (TEM). (A) BEI-inactivated, (B) 7.5% PEG precipitation, (C) 50% AS precipitation and (D) UF. Scale bar, 100 nm.

concentrated by the 100 or 300 kDa membrane was not significantly different (data not shown). For this reason, a 300 kDa membrane was used in UF to remove the NSP more efficiently. AS is also used for concentrating proteins because of its high salting-out efficiency, high solubility, and low price (Bollag and Edelstein, 1991). The FMDV concentration was carried out using 50% AS, based on previous studies (Booth et al., 1978; Brown and Cartwright, 1963).

Inactivated FMDV supernatants were concentrated 50 times with 7.5% PEG or 50% AS. On the other hand, the supernatants were concentrated 10 times by UF. BEI-inactivated supernatant was used as a control for the comparison among the three methods described above. The amount of 146S particles determined by spectrophotometry in the BEI-inactivated FMDV supernatant was 4.44 ± 0.14 μg/ml. PEG treated samples (3.70 ± 0.17 μg/ml) yielded more 146S particles than either AS treated samples (2.82 ± 0.11 μg/ml) or UF samples (1.49 ± 0.04 μg/ml) (Table 2). On the basis of these results, respective recovery was calculated as follows: PEG treated samples, 83.36 ± 1.81%; AS treated samples, 63.59 ± 2.72%; and UF samples, 33.50 ± 0.39% (Table 2). Concentrated and purified 146S particles of FMDV from each test group were confirmed to be intact based on a spherical shape and diameter of 25–30 nm by TEM (Fig. 1). In this study, inactivated FMDV concentrated by 7.5% PEG showed significantly higher antigen recovery than either 50% AS or UF (Table 2). Furthermore, total proteins and NSP content were also lowest in the 7.5% PEG precipitated samples and highest in the UF samples (Table 2). Taken together, 7.5% PEG precipitation is the most efficient method for FMD vaccine production, not only to concentrate FMDV with least antigen loss but also to purify 146S antigen with the least contamination by other unnecessary proteins such as NSPs.

In conclusion, on the basis of the present findings, it is suggested that 7.5% PEG precipitation is the best method of concentrating and purifying FMDV antigen for vaccine production with O-JC strain. To the author's knowledge, no previous reports have compared these common concentration methods or determined the optimal PEG concentration in regards to quantifying intact virus particles (146S), which is the most crucial factor to secure high FMD vaccine quality. Although different serotypes and strains will react potentially differently to these methods due to differences in biophysical stability, the present study is the first to provide valuable information not only to establish the domestic FMD vaccine manufacturing process in Korea, but also to develop a new vaccine production process using locally isolated FMDV strains in other countries.

Conflict of interest

None.

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