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Purification of Foot and mouth disease virus non-structural protein 3ABC from vaccine in-process samples and their characterization

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Abstract

Foot and mouth disease is a highly contagious disease that affects the cloven hoofed animals which includes livestock and can impact on nation's economy. Adjuvanted inactivated whole virus (140s) is used as vaccine to protect animals and control the disease. The vaccine production process is well established by many commercial manufacturers by culturing the virus on BHK cell line. The development of vaccine requires partial purification of the antigen batches to eliminate unwanted proteins along with virus non structural protein 3ABC to claim as a marker vaccine in differentiating vaccinated from infected animals (DIVA) and also to improve the quality of the vaccine thus enhancing the levels of protection. In the current study, we have shown the process of eliminating 3ABC protein from the antigen batches by size exclusion chromatography using Sephacryl S-400 and Toyopearl HW 65F columns. We have demonstrated the removal of 3ABC protein in various antigen batches of FMDV Serotype O by each of the above chromatographic media and quantifying the 3ABC protein by ELISA during the chromatographic process.The process involving the treatment of sample with the buffer containing Triton X -100 has shown a minimum NSP levels in the final antigen purified by both the chromatographic media. We also suggest that the method could also be applied for other serotypes of FMDV. The present method has the potential for a large scale production of marker vaccine which can be used in differentiating vaccinated from infected animals (DIVA).

Keywords: FMDV, NSP, Chromatography, ELISA.

Introduction

FMDV is a highly contagious disease affecting the domestic and wild cloven hoofed animals like sheep, cattle, goat, pig, deer, bison etc. It is causing severe economic loss to the developing countries, as majority of them are farming animals contributing to the nation's economical integrity. The virus belongs to the genus Apthovirus and family *picornaviridae*. It is prevalent and confined to a specific region with seven serotypes viz. Type O, A, C, Asia-1, SAT-1, SAT-2 and SAT-3 (1) and the spread of the disease can be prevented by vaccination.Till date the vaccine produced against FMD is a cell cultured derived and an inactivated whole virus.The potency of inactivated virus vaccines that are routinely used as a part of elimination of the disease are majorly dependent on virus integrity (2 and 3).

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The virus is a non-enveloped, icosahedral symmetry with 30nm diameter and composed of 60 copies of four structural proteins (VP3, VP1, VP4 and VP2) and a copy each of non-structural proteins L, 2A, 2B, 2C, 3A, 3C, 3D and three copies of 3B with their precursor forms 2AB, 3AB and 3ABC (4, 5, 6 and 7). Vaccine component against FMD is the inactivated intact whole virus, 140s particle with a molecular weight of 8200 KDa (8).The sucrose based density gradient fractionation method was considered as gold standard for the quantification of virus and determining the vaccine payload (9). Vaccine payload is determined by the quantity of the virus required to protect the animals from infection (10). Countries like India, endemic to the disease have national mass vaccination program to prevent and eliminate the disease. For a successful vaccination program and to export the vaccine to the FMD free countries, it is required to have a protective low dose PD_{50} value and the presence of lower NSPs (Non - Structural Proteins) level in the vaccine (11). Among NSPs, 3ABC is most immune dominant and hence can be used as the marker protein in differentiating infected from affected animals (DIVA) (12). The unavailability of a consistent method to estimate the active component present in vaccines is a vital factor to sustain the cost and to replace the complex tests that are involving the usage of large animals as a batch release criteria.

The chromatographic techniques are the traditional and conventional process being used in separation of the bio macromolecules (11). Advancement in the column chromatography has allowed the purification of diversified class of biomolecules including viruses in less time. There are evidences for purification and quantification of viruses based on ion exchange chromatography (12, 13, 14 and 15) and affinity chromatography (16, 17 and 18). FMDV has been purified and quantified by affinity chromatography (19) and receptor ligand binding of FMDV has been studied extensively (20). The virus can also be quantified using Size exclusion chromatography (SEC) as described by Yang, 2015 (21). To date preparative columns with pore size of stationary phase above 100 nm are commercially available, making it possible to analyze most of large molecules within the range of 10–100 nm. Moreover, SEC requires a mild operation unlike ion exchange or hydrophobic chromatography, wherein latter case there could be strong interaction between the antigen and the chromatographic media. Such a strong interaction may break up the virus integrity. As reported earlier, FMDV was quantified by SEC from clarified harvested virus culture in serum free media (22).

In the present study, we demonstrated the process of eliminating the virus non-structural proteins (NSPs) from the FMD inactivated and concentrated vaccine antigen batches by the application of size exclusion chromatography technique that has the potential to act as a marker vaccine in differentiating vaccinated from infected animals (DIVA). The presence of NSPs at various stages of purification is done by detecting and quantifying 3ABC protein.

Materials and Methods:

FMDV vaccine strain of serotype O was cultured on BHK cell line with MEM (Hi Media GmbH, Germany) containing 1% adult bovine serum (GIBCO, USA).The harvested virus was inactivated and concentrated using 500 KDa cut-off Hydrosart ultra-filtration cassettes (Sartorius stedim, USA), Ribonuclease A (Sigma, R 4875-100 mg), Hiprep Sephacryl S – 400 HR 16/60 (GE Healthcare, USA), Toyopearl HW65F(Tosoh biosciences, Japan) packed in XK 16/70 column (GE Healthcare, USA), Gradient maker (BIO-RAD, USA) and all other inorganic chemicals were procured from Merck, USA.

Preparation of Sample: The harvest and concentrated vaccine antigen batches of serotype O were centrifuged at 8000xg for 15 min and treated with equal volume of 0.25% v/v triton X 100 in 100mM Tris buffer (pH 7.6). Alternatively, the antigen were mixed with Triton X 100 with a final concentration of 0.125% v/v. The treated sample is then incubated at 4ºC for 2-3 h by gentle mixing.

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Size Exclusion Chromatography: The treated and untreated cultures were used for purification by column chromatography. The column was pre equilibrated with two column volumes of 50mM Tris and 150mM NaCl, pH 7.6 as mobile phase and both the virus batch preparations were incubated with 0.1% v/v of 1M RNase at room temperature (RT) for 30 min prior to loading onto the column. The sample was then loaded with 5mL loop volume (4% of total column volume) at 30cm/hour linear flow rate and a pressure limit of 0.15 MPa on Akta purifier chromatographic system (GE Healthcare, USA). The peak resolution was observed as absorbance (mAU) at 254nm with volume (mL) as baseline. The running conditions were kept similar for both the media i.e. Sephacryl S-400 and Toyopearl HW65F.

Quantification of the whole virus by Sucrose density gradient: The whole virus was quantified based on the conventional method by separating on sucrose density gradient centrifugation as described earlier (9).The excluded chromatographic fractions from the column were pooled and treated with ribonuclease A and the samples were overlaid on a 25–45% (v/v) sucrose concentration gradient prepared with a gradient maker (Model 485, BIO-RAD). The overlaid sample was centrifuged at 40000 rpm for 2 hrs. on HITACHI ultracentrifuge. Post centrifugation the fractionated gradients were scanned at 254 nm using UA-6 absorbance detectors (Teledyne Isco, USA) by pushing with 60% (v/v) sucrose solution.

Immunoblotting of the chromatographic fractions: The eluted fractions from the column chromatography samples were also analyzed by immunoblotting. The fractions along with the load sample were run on 12% denaturing polyacrylamide gels. Western blotting was done using nitrocellulose membrane (Immobilon, Amersham, USA) and probing with the bovine convalescent sera. 5% (w/v) skimmed milk (Difco, USA) in PBSA was used as blocking agent. Anti-bovine IgG conjugated with HRPO was used as secondary antibody (Sigma, USA). The blot is developed by activating the chromogen DAB substrate(3, 3'-diaminobenzidine tetrahy drochloride) with hydrogen peroxide.

Quantification of the 3ABC protein by ELISA: 3ABC content in the eluted fractions from the column chromatography were measured by in-house developed ELISA method.The 3ABC content in the sample will be captured between immobilized 3ABC specific mAb (8C4) and biotin labeled 8C4 in sandwich format. Bacterial expressed r3ABC antigen was used as the internal reference standard to estimate 3ABC content in the unknown samples.

Results:

Purification of antigen samples by Size Exclusion Chromatography:

The chromatography was carried out independently with six different antigen batches (Serotype O) in triplicates. Batch 1 and batch 2 refers to untreated ones, batch 3 and batch 4 refers to antigen batches that were treated with buffer containing Triton X100 and batch 5 batch 6 refers to the antigen batches that were directly treated with Triton X100.The chromatographs showed peak profile with a symmetrical peak corresponding to the column void volume at 42 and 52 mL when run with a volumetric flow rate of 1mL/min for Sephacryl S 400 and Toyopearl HW 65F respectively.The elution of the chromatography was collected as fractions with 2 mL each (Fig. 1-3). The fractions were then analyzed by western blot using 12% SDS- PAGE under denaturing conditions for both virus structural proteins and non-structural proteins.

Immunoblot analysis of the void volume fractions: The eluted fractions from the void volume were collected and analyzed by immunoblotting using bovine convalescent sera**.** We have pooled the eluted peak fractions into two halves prior to testing. The blot depicted the presence of band corresponding to virus structural protein VP1 at 34 KDa and no 3ABC specific band at 56 KDa detected in the fractions collected from batch 3 and 4 respectively, as shown in figure 4.

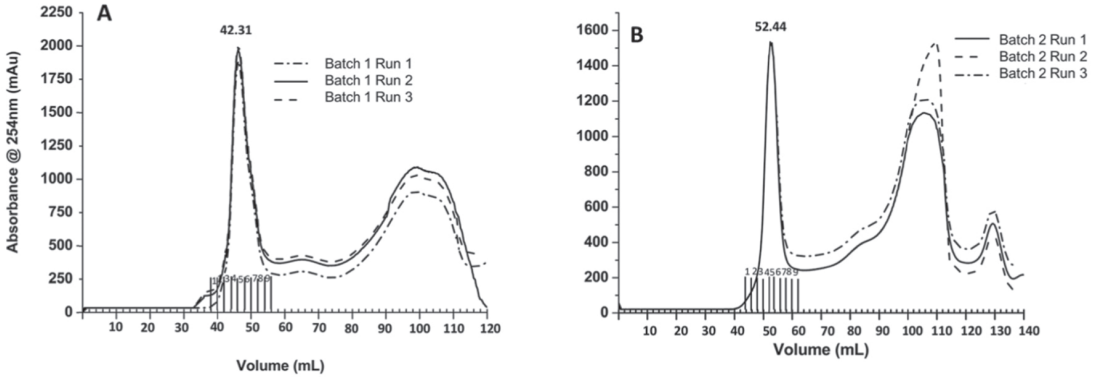

Fig. 1. Chromatogram showing three independent runs of the antigen batches loaded without treatment. A. Sephacryl S 400 and B. Toyopearl HW 65F. Note: The extended tick labels on X-axis indicates the fractions of the respective peak.

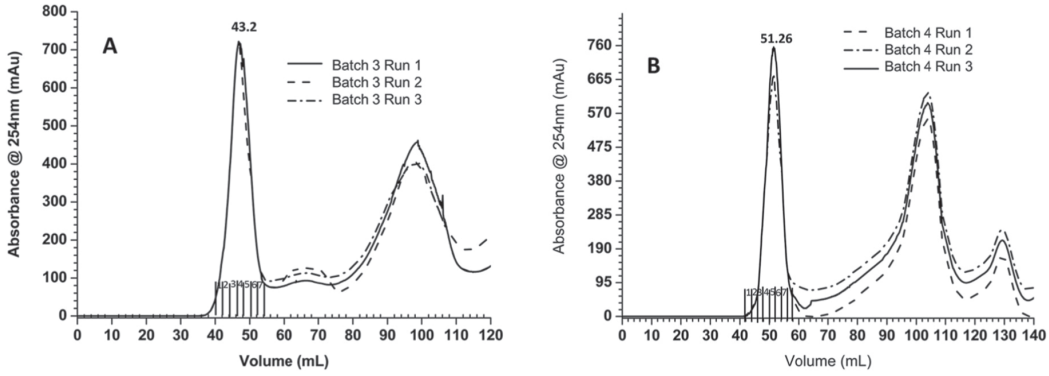

Fig. 2. Chromatogram showing three independent runs of the antigen batches loaded after treating with buffer containing 0.25% v/v Triton X 100. A. Sephacryl S 400 and B. Toyopearl HW 65F. Note: The extended tick labels on X-axis indicates the fractions of the respective peak.

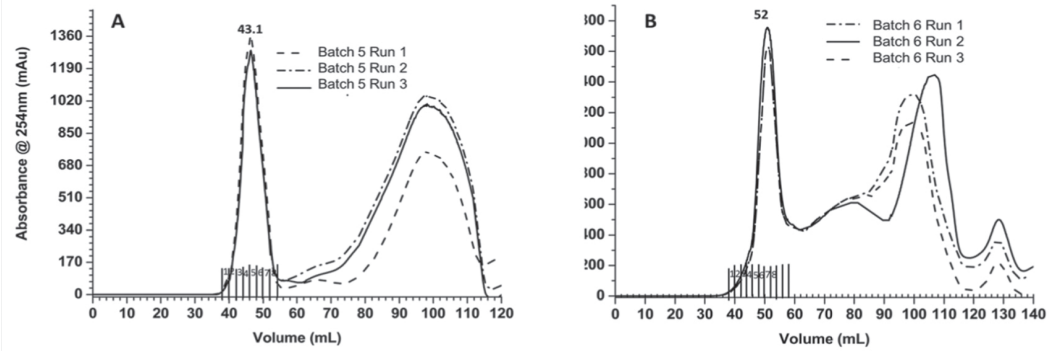

Fig. 3. Chromatogram showing three independent runs of the antigen batches loaded after treating with 0.25% v/v Triton X 100. A. Sephacryl S 400 and B. Toyopearl HW 65. Note: The extended tick labels on X-axis indicates the fractions of the respective peak.

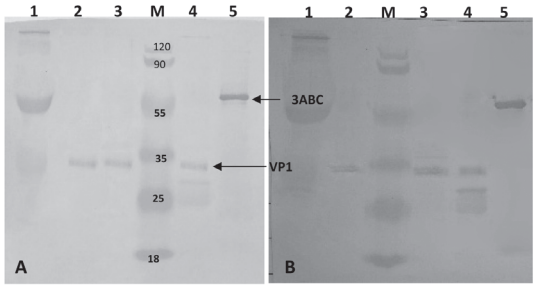

Fig. 4. Western blot analysis of the eluted fractions probed with bovine convalescent sera A. Batch 3 and B. Batch 4, Lanes: 1. Load 2. First half pooled fractions 3. Second half pooled fractions 4. Purified 140s 5. r3ABC.

Quantification of the 3ABC protein by ELISA:

The eluted void volume fractions were analyzed by quantifying 3ABC protein through sandwich ELISA method developed earlier in our lab. All the fractions from all the three repeat runs were analyzed. Figure 5, a multi curve double Y axis plot (drawn using Origin version 8.5 data analysis software), indicates percentage removal of 3ABC in micrograms before and after chromatography. The percentage removal of 3ABC from batches 3 and 4 were found to be 94% and 100 % respectively.

Quantification of the whole virus by Sucrose density gradient: The aliquots from each eluted fractions were pooled that would represent the entire void volume. The 140s (whole virus) content in the pooled fraction were quantified by separating on density based sucrose gradient. Treated sample (Batch 3, 4, 5 and 6) and untreated samples (Batch 1 and 2) did not show any significant loss.The recovery values after chromatography were above 80% for all the runs with relatively higher recoveries (Table 1) for the batch 1 and batch 2 samples.

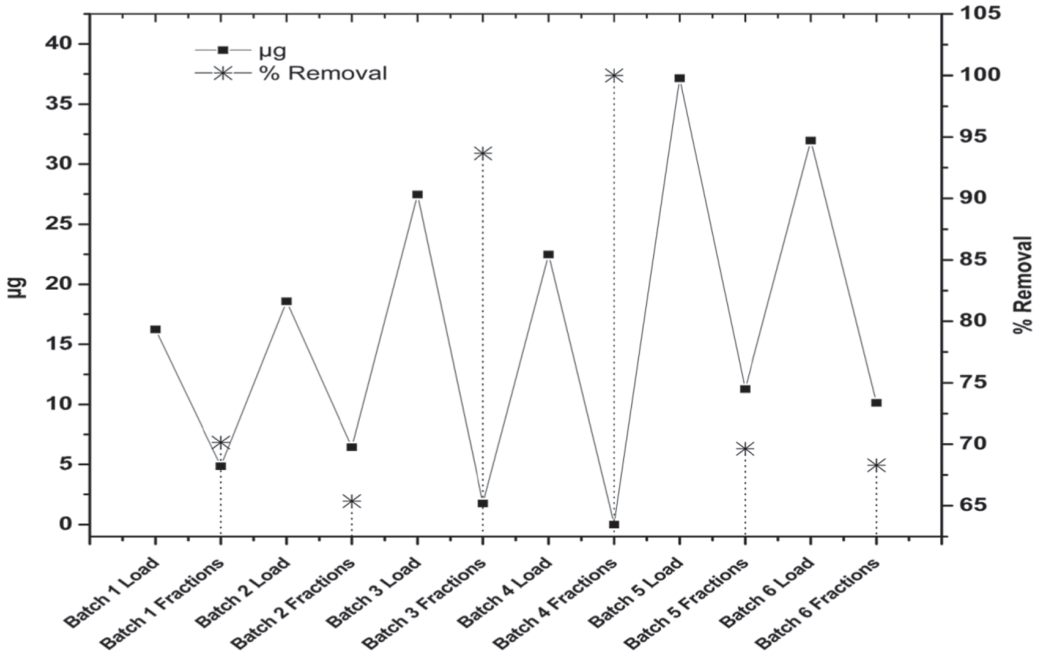

Fig. 5. Double Y-axis plot showing the quantity of 3ABC on the left Y-axis and their percentage removal of the respective runs on the right Y-axis.

Table 1: 140s quantification of the in process samples showing percentage recoveries

Pre-treatment of sample	Run	140s value (µg/mL) post sample treatment	140s value (µg/mL)	Total 140s injected (µg/5mL)	Eluted void volume (mL)	140s value (µg/mL) after chromato graphy	Total 140s (µg) after chromato graphy	% Re-covery
Untreated	Batch 1	65	NA	325	18	15	270	83.1
	Batch 2	51.6	NA	257.8	18	12	216	83.8
Treated	Batch 3	44	22	110	14	6.4	89.6	81.4
	Batch 4	50	25	125	14	7.2	100.8	80.6
	Batch 5	42	42	210	16	11.3	180.8	86.1
	Batch 6	54	54	270	16	13.6	217.6	80.6

Discussion:

Foot-and-mouth disease (FMD) is one of the known devastating viral disease of cloven-hoofed animals. It shows significant socio economic consequences worldwide, from national livestock industries declining due to international trade restrictions, affecting the stock productivity and livelihood. The primary method of controlling the disease in endemic areas is only by way of regular vaccination with inactivated vaccine. However, there are many limitations in the manufacturing of NSP free vaccine thus interfere in sero diagnosis of animals in differentiating infected from vaccinated animals (11 and 23).

Attempts are being made to standardize the production process of a vaccine that is operator independent and can be automated. One such approach is the use of separation and purification techniques like chromatography in vaccine manufacturing process. Since FMDV is a pH labile virus (24 and 25) and there is no reported PI of the whole virus (26). Purification of virus from the concentrated cell culture harvest becomes strenuous by Ion-exchange chromatography. The affinity purification of FMDV though reported earlier (19), has limitations in distinguishing the intact antigens from disassembled small particles and the large aggregates.The method reported also involves sensitive procedure involving FMDV labile parameters and difficult to adopt in large scale.The method described by Spitteler, 2011 (22), showed that concentration of inactivated FMDV could be estimated by SEC in vaccine manufacturing process.

In the current study, we have optimized the process for removal of viral NSPs along with other discrete proteins in the concentrated virus culture. The purified virus is eluted in the void volume using two different size exclusion chromatographic separation media, Sephacryl S-400 and Toyopearl HW65F. We have selected the chromatographic media based on their exclusion limit of 9000 KDa and more than 5000 KDa for Sephacryl S-400 and Toyopearl HW 65F respectively. The estimation of 140s through sucrose density gradient has shown a total recovery of more than 80% and the content of 3ABC protein in the purified virus concentration were reported to contain less than 6% w/w. The purification process can be validated further by immunizing animals and testing for the sero conversion against 3ABC NSP according to the OIE guidelines. This strategy will be a useful tool for other serotypes also and this method has the potential for a large scale manufacturing of NSP free FMD vaccine.

Conclusion:

Currently available conventional FMD vaccine prepared with virus grown on mammalian cells consists of unwanted moieties which interferes in production of quality vaccine. With an objective of developing a quality FMD vaccine,

we have come up with the method of partial purification of the antigen batches by chromatography. We have incorporated chromatography step in the purification process either by using Toyopearl HW 65F or Sephacryl S 400 as chromatographic media, which has effectively contributed in the removal of FMDV NSP (3ABC) at 95% and 100% respectively. The process also involved treatment of the sample with Triton X-100 for solubilization of the membrane proteins prior to chromatography. Presently developed method has the potential in production of NSP free quality vaccine that could act as marker vaccine in DIVA.

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